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UNIVERSITY OF ALBERTA

Synthesis of Substrate and Transition State Analogues of Diaminopimelic Acid, Isolation and Inhibition Studies on DAP Epimerase

by

Kanwar Pal Singh Sidhu

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE

Department of Chemistry

EDMONTON, ALBERTA

Spring 1993

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Synthesis of Substrate and Transition State Analogues of Diaminopimelic Acid, Isolation and Inhibition Studies on DAP Epimerase submitted by Kanwar Pal Singh Sidhu in partial fulfilment of the requirements for the degree of Master of Science.

.....

Dr. John C. Vederas (Supervisor)

.....

Dr. Monica M. Palcic

.....

Dr. Michael A. Pickard

Date: December 21, 1992

To my parents

ABSTRACT

Diaminopimelic acid (DAP), an essential component of peptidoglycan in bacterial cell walls, is an attractive target for rational design of new antibacterial agents. DAP analogues which inhibit enzymes in the biosynthetic pathway of this amino acid may be broad spectrum antibiotics that are non-toxic to mammals. An imidazole analogue of DAP, designed as a potential transition state mimic, was synthesized by nucleophilic attack of benzyl imidazole-4-carboxylate on *N*-CBz-L-serine β-lactone to give (2S)-2-(*N*-Benzyloxycarbonyl)amino-3-(5-benzyloxycarbonyl-1-imidazolyl) propionic Acid **11a** and its regioisomer, (2S)-2-(*N*-Benzyloxycarbonyl)amino-3-(4-benzyloxycarbonyl-1-imidazolyl)-propionic Acid **11b**, both of which were deprotected to the free amino acids (2S)-2-Amino-3-(5-carboxy-1-imidazolyl)propionic Acid **5a** and (2S)-2-Amino-3-(4-carboxy-1-imidazolyl)propionic Acid **5b**, respectively. The structure of **11b** was verified by X-ray crystallographic analysis. DAP epimerase was isolated from an over-producing clone of *Escherichia coli* LE134, and **5a** and **5b** were tested as potential inhibitors of it and of DAP dehydrogenase from *Bacillus sphaericus*. Neither compound was a substrate of either enzyme or showed significant inhibition.

An isoxazoline analogue of DAP was synthesized by 1, 3-dipolar cycloaddition of carboxymethylformonitrile oxide, generated in situ from methyl glycinate to methyl (2*S*)-2-(*N*-benzyloxycarbonyl)aminopentenoate to give Methyl (2S)-2-(N-Benzyloxycarbonyl)amino-3-(3-carbomethoxy-2-isoxazolin-5-yl) propionate **21a** and its diastereomer Methyl (2S)-2-(N-Benzyloxycarbonyl)amino-3-(3-carbomethoxy-2-isoxazolin-5-yl)propionate **21b**. Their structural isomers **22a** and **22b** were also isolated in minor amounts. Deprotection of **21a** and **21b** gave the free amino acids (2S)-2-Amino-3-(3-carboxy-2-isoxazolin-5-yl)propionic Acid **4a** and (2S)-2-Amino-3-(3-carboxy-2-isoxazolin-5-yl)propionic Acid **4b**, respectively. Studies with DAP epimerase indicated that **4a** and **4b** were

not substrates of the enzyme and showed weak competitive inhibition. However, **4b** was a good competitive inhibitor (20 μM) of DAP dehydrogenase.

N-Substituted diaminopimelates, 2-(*N*-o-Nitrophenylsulfenyl)diaminopimelic Acid **1**, 2-(*N*-o-Nitrophenylsulfinyl)diaminopimelic Acid **2** and 2-(*N*-o-Nitrophenylsulfonyl)diaminopimelic Acid **3** were synthesized as a mixture of all possible isomers and tested against DAP dehydrogenase and DAP epimerase. None of them showed any significant inhibition of DAP dehydrogenase or epimerase.

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LIST OF ABBREVIATIONS

D-Ala	D-Alanine
L-Ala	L-Alanine
AZIDAP	2-(4-Amino-4-carboxybutyl)-2-aziridine carboxylic acid
APT	Attached proton test
Cys	Cysteine
CI-MS	Chemical ionization mass spectrometry
DAP	Diaminopimelic acid
DTT	Dithiothreitol
DE-52	Diethylaminoethyl cellulose
DMSO	Dimethyl sulfoxide
DMAD	Dimethyl azodicarboxylate
EDTA	Ethylenediaminetetraacetic acid
HPLC	High performance liquid chromatography
IR	Infrared spectroscopy
J	Coupling constant
kDa	Kilodalton
K_m	Michaelis constant
MS (POSFAB)	Mass Spectrometry (positive fast atom bombardment)
mCi	millicurie
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide hydrogen phosphate
Nuc	Nucleophile

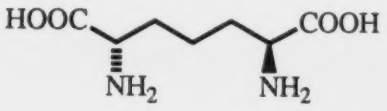
NMR	Nuclear magnetic resonance
PMSF	Phenylmethylsulfonyl fluoride
Rpm	Revolutions per minute
TLC	Thin layer chromatography
THF	Tetrahydrofuran
Tris.HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TSP	3-(Trimethylsilyl)-1-propanesulfonate
UDP-GlcNAc	Uridine diphosphate-*N*-acetyl glucosamine
U	Unit of activity
CBz	Benzyloxycarbonyl

1. INTRODUCTION

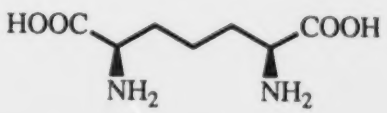
1.1 Occurrence and significance of diaminopimelic acid

Diaminopimelic acid (DAP) is a naturally occurring amino acid which is synthesized in bacteria and higher plants.[1] This diamino diacid, has raised enormous interest ever since its discovery in *Corynebacterium diphtheriae* almost 40 years ago.[2,3]

DAP serves as a vital component of peptidoglycan in the cell wall of virtually all Gram negative and mycobacteria, and in some Gram positive bacteria.[4-9] *meso*-Diaminopimelic acid is the most widely encountered stereoisomer, although the LL- and DD- isomers have also been found in peptidoglycan of certain species.[10]



LL-DAP



***meso* -DAP**

LL-Diaminopimelic acid and *meso*-diaminopimelic acid are also involved in the last two steps in the biosynthetic pathway of the amino acid, L-lysine, in bacteria.[11-13]

One further role of DAP is to serve as an anchor to the cell wall in various membrane associated macromolecules such as lipoproteins, where approximately every tenth peptide subunit is linked by the α-carbonyl group at the L-centre of diaminopimelic acid to an ε-amino group of a *C*-terminal lysine residue of the lipoprotein.[14,15]

1.2 Peptidoglycan: structure and biosynthesis

Peptidoglycan is a network like structure which provides strength and shape to the bacterial cell wall. In Gram positive bacteria, peptidoglycan may account for almost 80% of the total weight of the cell wall, whereas in Gram negative bacteria it accounts for only about 10% of this weight.[8] The structure of peptidoglycan can be arbitrarily divided into three parts : the glycan backbone; the linear muramyl peptide and the peptide cross-link (Figure 1). The glycan chain consists of alternating residues of *N*-acetyl glucosamine and *N*-acetyl muramic acid. A β(1-4) linkage between the aminosugars has been found in all bacteria.[16] The D-lactyl group of *N*-acetyl muramic acid bears a short tetrapeptide composed of L-Ala-γ-D-Glu-*meso*-DAP-D-Ala. Variation in the composition of the tetrapeptide has been observed in both the Gram negative and Gram positive bacteria. Gram negative bacteria always contain *meso*-DAP as the third amino acid of the tetrapeptide, whereas in many Gram positive bacteria it is replaced by L-lysine, L-ornithine or L-homoserine.[8] L-serine has also been found as the first amino-acid in the tetrapeptide of *Eubacterium limosum* .[8]

The peptide units are involved in cross-linking between the *C*-terminal D-alanine of one peptide to the ω-amino group of a DAP residue of another peptide. Variation in the above pattern of cross-linkage has been observed in many Gram positive bacteria.[17] This cross-linking creates a covalent network that has great mechanical strength as well as considerable elasticity. The extent to which cross-linkage occurs varies from one species to another.[17] Peptides which do not become involved in the cross-linkage often lose one or both terminal D-alanine residues by the action of carboxypeptidase.[16]

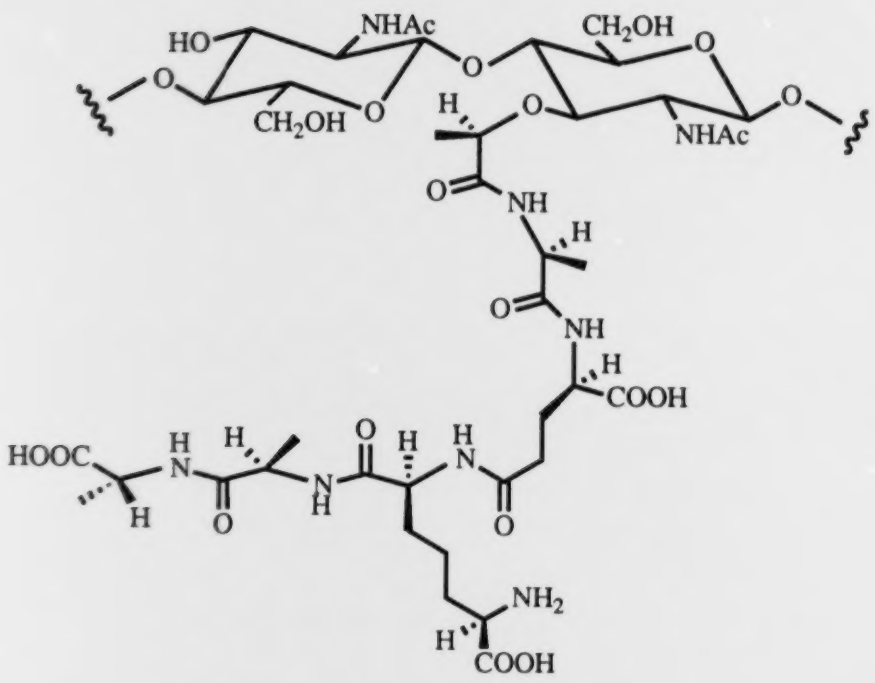


Figure 1: Structure of peptidoglycan monomer from *Escherichia coli*

Biosynthesis of this highly complex and unusual macromolecule is based on the synthesis of two uridine nucleotides, UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylmuramyl-L-Ala-D-*iso*-Glu-*meso*-DAP-D-Ala-D-Ala (UDP-MurNAc-pentapeptide), which act as soluble precursors of peptidoglycan. Further action by a series of enzymes results in the coupling and polymerization of the disaccharide pentapeptide subunits.[12] Transpeptidation of the polymerized subunits outside the cytoplasmic membrane generates mature insoluble peptidoglycan.[14-16]

1.3 Lysine: significance and biosynthesis

Lysine is a building block of bacterial cell wall peptidoglycan of some bacteria and involves LL-DAP and *meso*-DAP as intermediates in its biosynthetic pathway.[18-21] Lysine, an essential amino acid, is not biosynthesised in mammals, necessitating its dietary uptake.[22] The absence of lysine biosynthesis in mammals provides a possibility of killing bacteria selectively, through the disruption of this pathway, by agents which would be predicted to have low toxicity in mammals. Since, DAP is not metabolised by mammals, antibacterial analogues of DAP may behave similarly.

Lysine production from aspartate is initiated by a phosphorylation catalyzed by aspartate kinase,[23] (Figure 2) the product of which is reduced to 3-aspartyl semialdehyde by aspartate semialdehyde dehydrogenase.[24] The semialdehyde reacts with pyruvic acid in an aldol condensation catalyzed by dihydrodipicolinate synthase.[25] The 2,3-dihydrodipicolinic acid obtained is reduced by dihydrodipicolinate reductase[26] and then ring opened with amino group succinylation by tetrahydrodipicolinate succinylase.[27] Higher plants and some bacteria use a different acylating agent, namely acetyl coenzyme A, rather than succinyl coenzyme A.[28] Transamination by succinyl diaminopimelate aminotransferase[27] followed by desuccinylation by succinyl diaminopimelate desuccinylase[29] provides LL-diaminopimelic acid. Epimerisation by diaminopimelate epimerase[30] results in the formation of *meso*-diaminopimelic acid. In the final step diaminopimelate decarboxylase[31] converts *meso*- DAP to L-lysine.

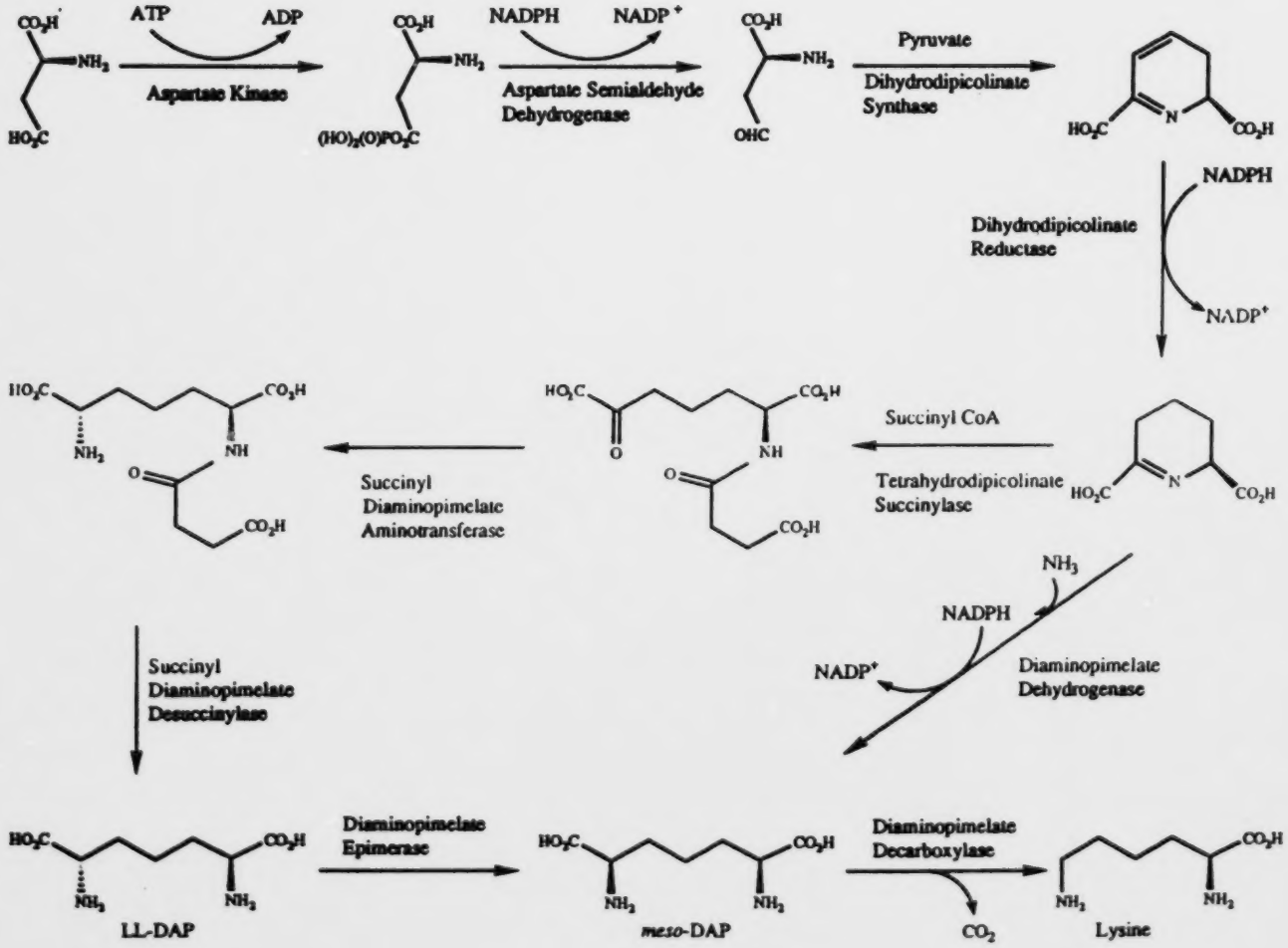


Figure 2: Lysine biosynthesis

Variations in the DAP pathway have been observed in some species, wherein tetrahydrodipicolinate is converted to *meso*-DAP in a single enzymatic step catalyzed by *meso*-diaminopimelate dehydrogenase.[32-34] Some species such as *Escherichia coli* and *Bacillus megaterium* have been found to possess the diaminopimelate epimerase but lack the diaminopimelate dehydrogenase, whereas others such as *Bacillus sphaericus* possess the diaminopimelate dehydrogenase but not the epimerase. It is also possible for both the enzymes to be present in one species, as in *Bacillus maceraus* and *Corynebacterium glutamicum*, although the activity of dehydrogenase is much higher than that of epimerase.[12]

1.4 Diaminopimelate epimerase and its mechanism

Diaminopimelate epimerase, despite being discovered several years ago, has only recently been purified from *E.coli*. [30] It was reported as a monomer with molecular weight of 34 000, although recently it has been suggested that the active form may be a dimer with identical subunits.[35] Interestingly, this epimerase does not require pyridoxal phosphate or another cofactor for its enzymatic activity. Probing of the active site, using 2-(4-amino-4-carboxylbutyl)-2-aziridine carboxylic acid (AZIDAP)[36] a time dependent irreversible inhibitor, indicated the presence of Cys-73 in the enzyme active site. A mechanism similar to that of proline racemase[37,38] has been proposed with the active site thiol group acting as one of the two groups involved in a deprotonation/protonation sequence resulting in epimerisation (Figure 3).[35] Based on this mechanism, DAP epimerase inhibition by AZIDAP has been proposed to occur as shown in Figure 4.

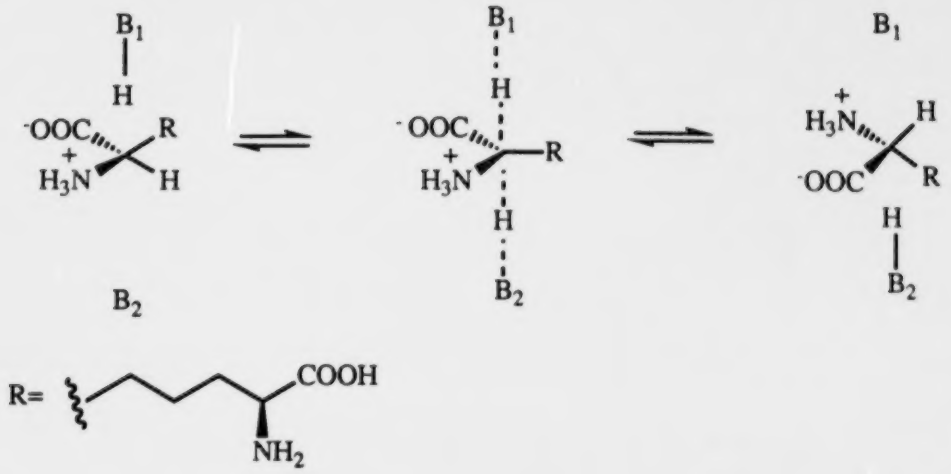


Figure 3: General representation of a proposed mechanism of DAP epimerase

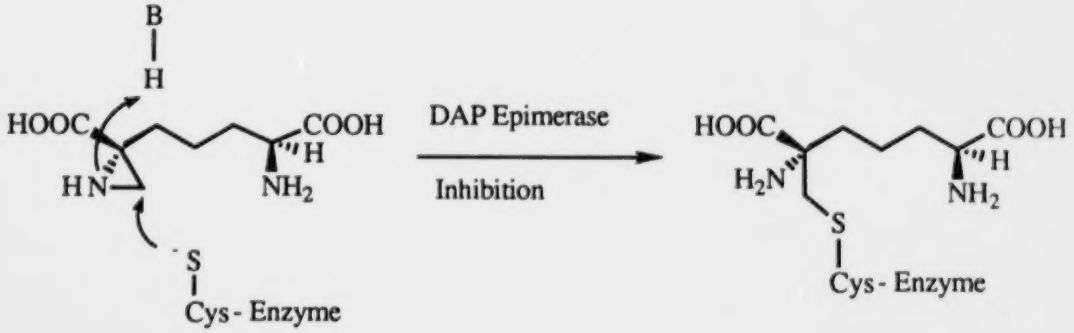


Figure 4: Concerted mechanism of AZIDAP inhibition of DAP epimerase

1.5 Diaminopimelate dehydrogenase: the alternative lysine pathway

meso-α,ε-Diaminopimelate D-dehydrogenase, a pyridine nucleotide-dependent enzyme, catalyses the reversible oxidative deamination of *meso*-DAP to tetrahydrodipicolinic acid.[32,33] This constitutes the alternative lysine pathway in several bacteria, predominantly in strains that are known as good lysine producers such as *B. sphaericus*, *Brevibacterium sp.*, *Corynebacterium glutamicum* and *Proteus vulgaris*.[39-42] The enzyme is composed of

two identical subunits each with molecular weight of *ca.* 40 000. It is substrate and cofactor specific, and utilizes only *meso*-DAP and $NADP^+$.[43,44] The amino group with D-configuration in the substrate is involved in the oxidative deamination process. This nicotinamide nucleotide-dependent dehydrogenase is known to be B-stereospecific, that is, the *pro-S* hydrogen at the C-4 of the dihydronicotinamide ring of NADPH is the one that is transferred to the substrate.[45]

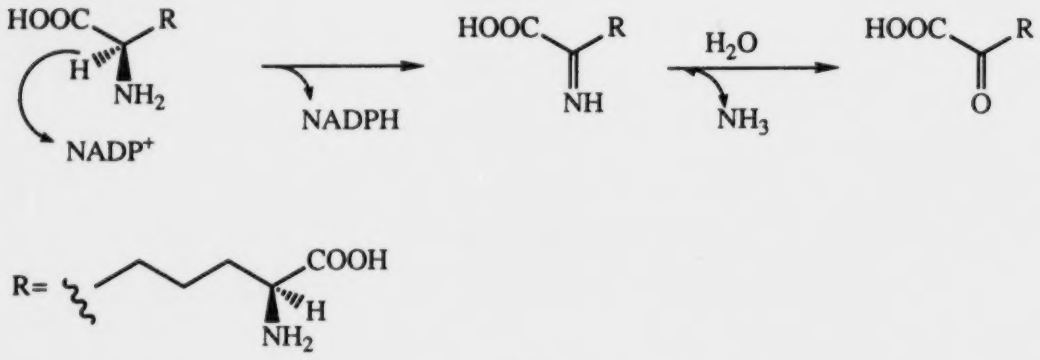


Figure 5: General representation of the mechanism of DAP dehydrogenase.

1.6 DAP analogues: approach and inferences drawn from the substrate mimics

Recent efforts in our research group to synthesize substrate analogues of DAP generated a number of potential inhibitors of DAP epimerase (Figure 6). The only known irreversible inhibitor of DAP epimerase is 2-(4-amino-4-carboxylbutyl)-2-aziridine carboxylic acid (AZIDAP) (Figure 4).[36] The *N*-hydroxy diaminopimelic acid **A** (K_i =5.6 μM for the mixture of isomers)[46] and β–fluorodiaminopimelic acid **C-F** (IC_{50} =4, 10, 25, 8 μM, respectively)[47] are potent competitive inhibitors of DAP epimerase. The phosphonic acid analogues of diaminopimelic acid **G-K**, wherein the carboxyl group of diaminopimelic acid

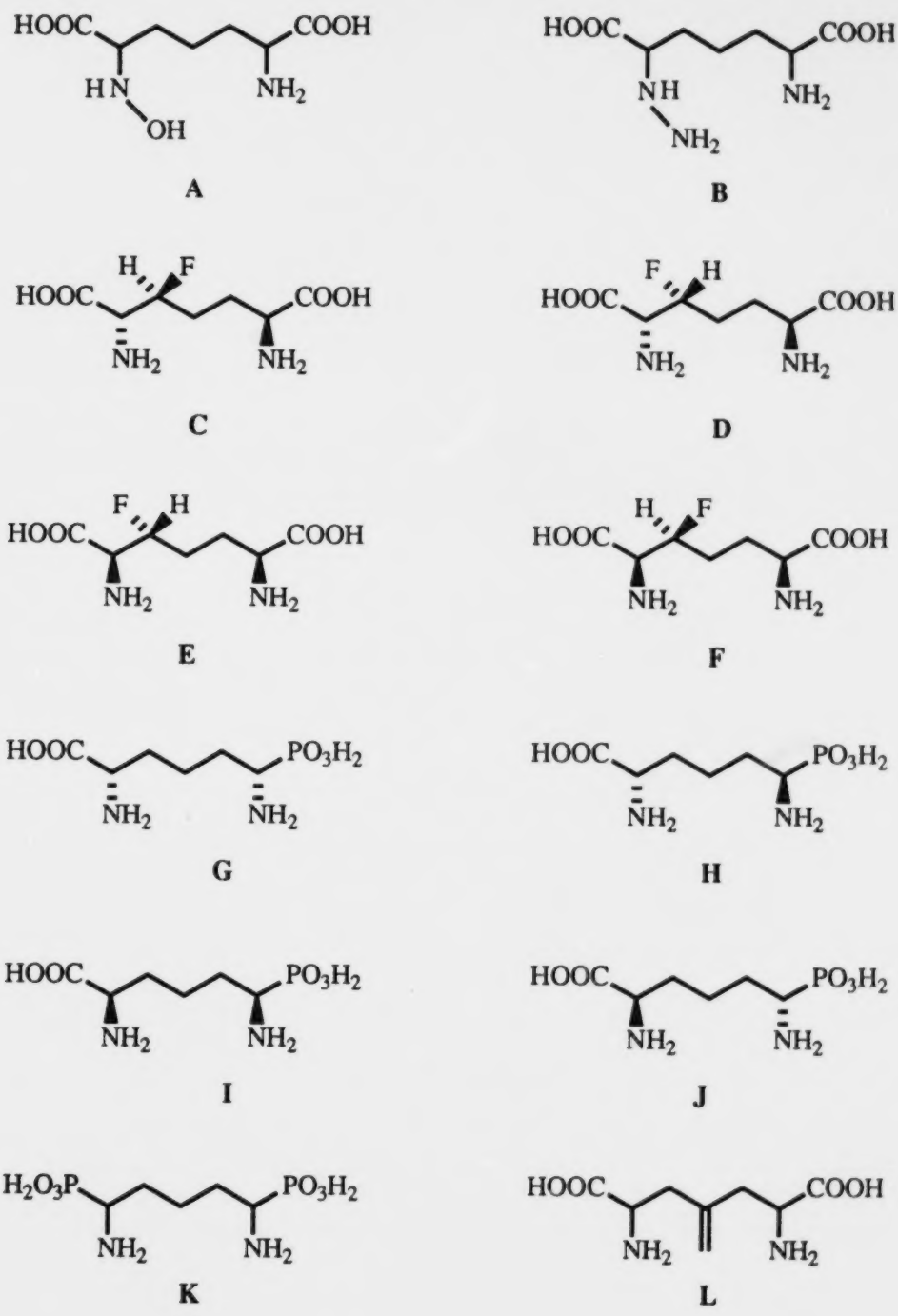


Figure 6: Substrate analogues of DAP

is replaced by a phosphonate group,[48] (**G**, **H** and **J**, K_i =3.9, 9.3 and 7.2 mM, respectively) are either weak competitive inhibitors or have no inhibitory effects against epimerase (**I** and **K**).[49,50] *N*-Aminodiaminopimelic acid **B** (K_i =2.9 mM)[46] and 4-methylene diaminopimelic acid **G**[51] were also tested as possible inhibitors because of their antibiotic activity, but were only modest competitive inhibitors of DAP epimerase.

These studies and use of other analogues[50] define certain substrate requirements for DAP epimerase. The enzyme requires both acidic and both basic groups in the substrate which must also have the seven carbon chain length of DAP. There is a very specific requirement of L-configuration at the distal end of the substrate. The reaction site may have L or D configuration.[30,47] Potent inhibition by *N*-hydroxydiaminopimelic acid **A** (Figure 6)

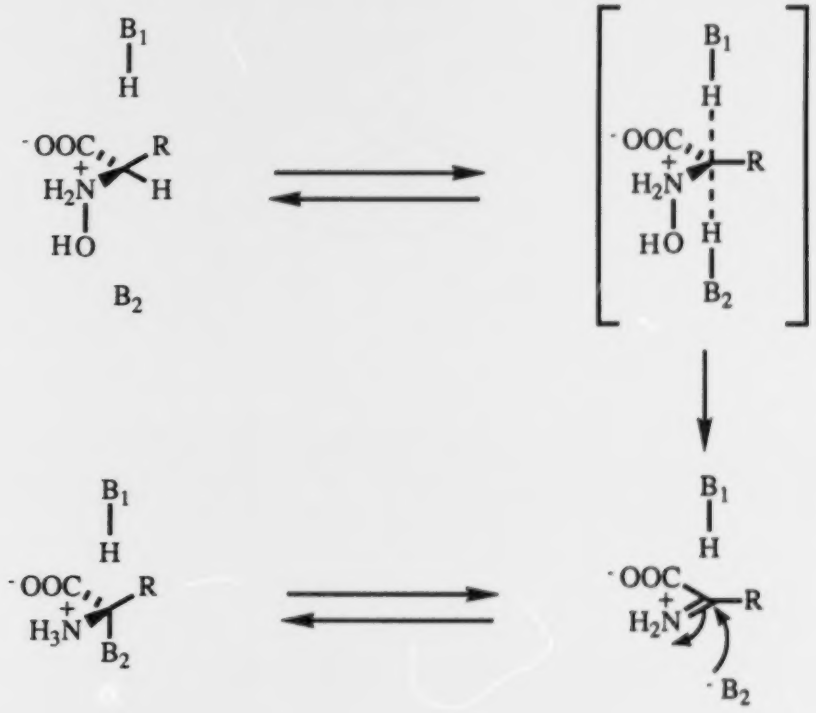


Figure 7: Proposed mechanism for inhibition of DAP epimerase by N-hydroxy DAP

suggests that its interaction with DAP epimerase may involve the elimination of the hydroxy group to generate an α-imine.[46]

This planar transition state intermediate could then possibly be attacked by the active site thiol group. Generation of a planar transition state would seem plausible and in agreement with the proposed carbanionic mechanism of DAP epimerase, which involves the generation of an sp^2 hybridized center at the α-carbon.[30,35]

Studies on the β-fluoro-DAP analogues **C-F** (Figure 6) also indicate generation of carbanionic character at the α-carbon which leads to the loss of HF. This elimination reaction and the inhibitory properties are dependent on the stereochemistry, presumably due to conformational orientation on the enzyme surface.[47] Of the four fluoro DAP isomers, **C** and **F** are more potent inhibitors than **D** and **E**. Since the rate of HF elimination is much more rapid for **D** and **E**, it appears that inhibition is due to the intact fluoro DAP which binds tightly to the epimerase. Interestingly, the mono- and diphosphonate diaminopimelic acids **G-K** (Figure 6) do not significantly inhibit DAP epimerase. Differences in bond length and/or ionization of the acidic group may account for the lack of binding to the enzyme active site.

1.7 Goal of the project and rationale for the design of the analogues

In order to develop an effective inhibitor of DAP epimerase and to obtain a better insight into the substrate specificity and properties of the enzyme, this project targeted synthesis of new transition state analogues of DAP. The rationale for the design of these analogues was based on: maintaining the length of the carbon chain; the substrate stereochemical requirements which demand that the distal carbon center be L; placing a planar geometry at

the α-carbon to mimic the epimerase transition state; and ensuring some conformational rigidity to increase binding of the analogue to the active site of the enzyme.

Two types of potential inhibitors were targeted. The first groups were *N*-substituted diaminopimelates bearing an *o*-nitrosulfenyl, *o*-nitrosulfinyl or *o*-nitrosulfonyl group (compounds **1, 2** and **3**, respectively) (Figure 8). Since DAP epimerase can eliminate the extra substituent from *N*-hydroxy DAP[46] and β-fluoro DAP,[47] it appeared possible that **1**, **2**, or **3** could potentially behave similarly. Thus, loss of the α-hydrogen and concomitant generation of negative charge at the α-carbon could in principle lead to the formation of an imine and loss of thiol, sulfenic acid, or sulfinic acid.

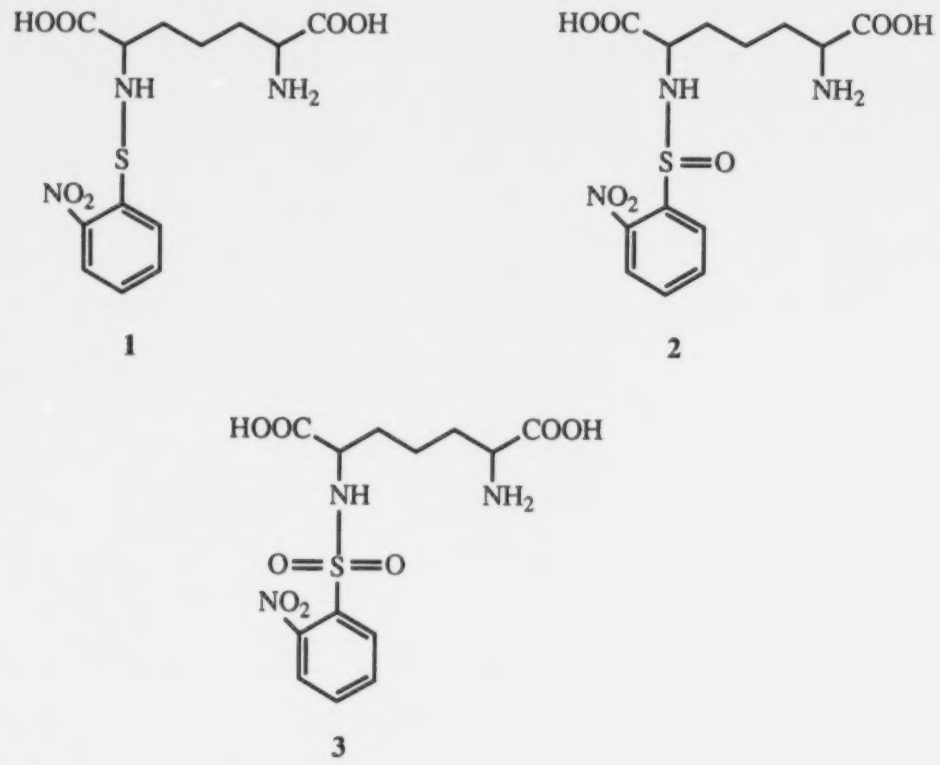
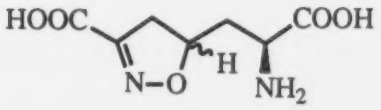


Figure 8: Sulfur containing target derivatives of DAP

The *ortho*-nitro substituent is present for several reasons. It is a powerful electron withdrawing group which could assist the elimination process.[52] In addition, *o*-nitrosulfenyl groups are present in a number of antimicrobial agents.[53] Finally, the *o*-nitro group enhances the aqueous stability of the corresponding aryl sulfenyl chloride,[54] thereby allowing it to react successfully with the nitrogen of amino acids, which are only soluble in water.

The potent inhibition of the enzyme, proline racemase, by pyrrolecarboxylic acid[38] was suggested to be due to the planar configuration at the α-carbon which resembles the transition state. Since a mechanism similar to proline racemase has been proposed for DAP epimerase, it seems that planar heterocyclic analogues, with structural similarity to DAP, may act in a corresponding manner. The isoxazoline analogue **4** and the imidazole analogue **5** are planar at the α-carbon and may therefore resemble the transition state of the epimerase. Similarly, they may mimic intermediates (eg. imine, α-keto acid) in the process catalyzed by DAP dehydrogenase. Both **4** and **5** are conformationally more restricted than DAP, and therefore could allow probing of substrate conformation in the active site of the epimerase and the dehydrogenase enzymes.



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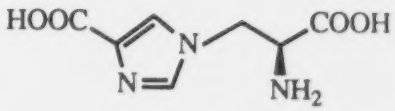


5

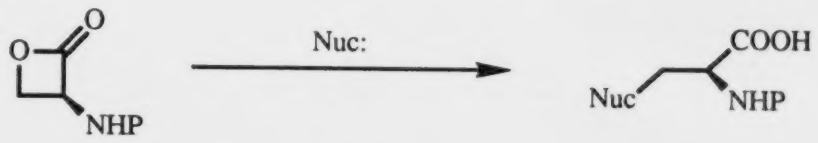
Figure 9: Potential isoxazoline and imidazole analogues of DAP

A number of isoxazoline and imidazole containing amino acids occur in nature and have interesting biological activities. For example, (α*S*,5*S*)-α-amino-3-chloro-4,5-dihydroisoxazole-5-acetic acid (AT-125), an antimetabolite from *Streptomyces sviceus*, exhibits significant antibacterial and antitumor activity.[55-58] Some of its analogues also display such effects.[59] α-Amino-β-(1-imidazolyl)propionic acid (isohistidine) has been isolated from natural sources.[60] 2-Nitroimidazole (azomycin) is a naturally occurring antibiotic,[61,62] and several other 2-nitroimidazole derivatives have been found to possess useful biological activities.[63-65] These precedents suggest that compounds **4** and **5** could also show unexpected but interesting effects in vivo.

RESULTS AND DISCUSSION

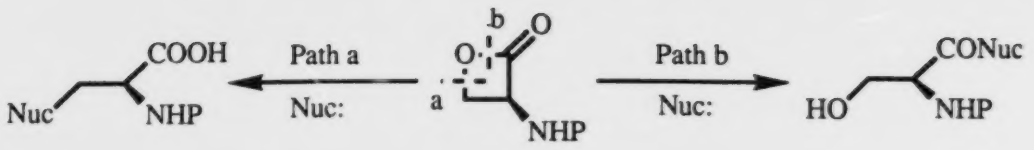
2.1 Synthesis of an imidazole analogue of DAP

Synthesis of the imidazole analogue **5** of DAP was based on methodology developed in our research group which demonstrated that β-substituted α-amino acids are available by ring opening of *N*-protected α–amino–β–propiolactone by carbon and heteroatom nucleophiles.[66,67]



P= Protecting Group

Nucleophilic attack on the β-lactone ring can proceed in two directions (Figure 10), namely alkyl oxygen cleavage and acyl oxygen cleavage. Various 'soft' nucleophiles can attack the β- methylene group in an SN2 manner resulting in an alkyl oxygen cleavage, whereas 'hard' nucleophiles like hydroxides, alkoxide and organolithium species attack at the carbonyl position causing an acyl oxygen cleavage.[67] For example, pyrazole attacks the lactone to give *N*-(benzyloxycarbonyl)-β-(pyrazol-1-yl)-L-alanine in 71% yield.[68] Hence it appeared that other heterocycles, such as imidazole, could also open the β-lactone ring to generate the corresponding amino acids.



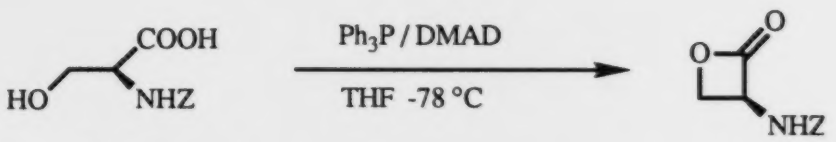
P= Protecting Group

Nuc= Nucleophile

Figure 10: Nucleophilic attack on β-lactone ring.

Synthesis of *N*-protected α-amino acid-β-lactones by cyclisation of serine derivatives can in principle be achieved by several approaches including carbonyl activation with carbodiimide reagants,[69] and generation of a leaving group at the β-position of the amino acid.[70] However, these methods give only low to modest yields, whereas hydroxyl group activation[71] using modified Mitsunobu conditions affords good yields.

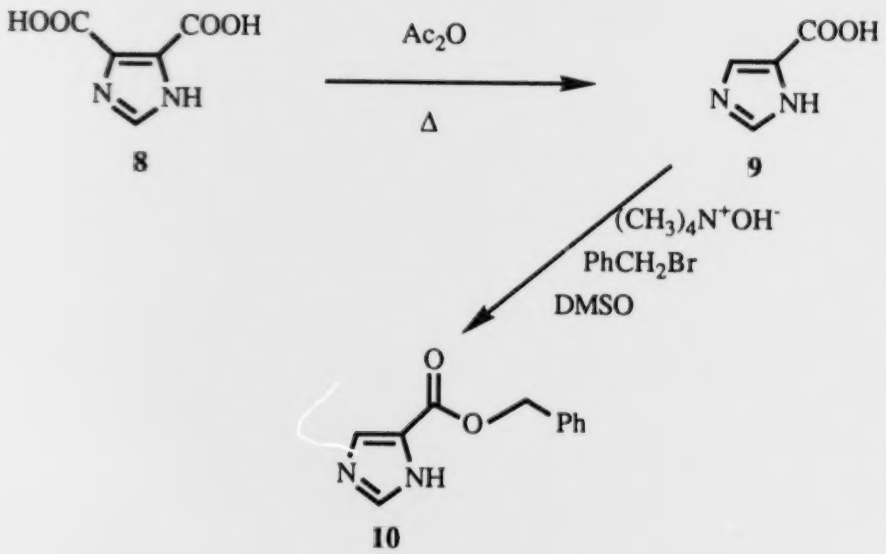
Reaction of triphenylphosphine and dimethyl azodicarboxylate (DMAD) at -78 °C results in the formation of an adduct [72] which upon addition of optically pure *N*-benzyloxycarbonyl-L-serine **6** generates the protected α–amino-β-propiolactone **7** in 60-72% yield without racemisation.[66,67]



6 **7**

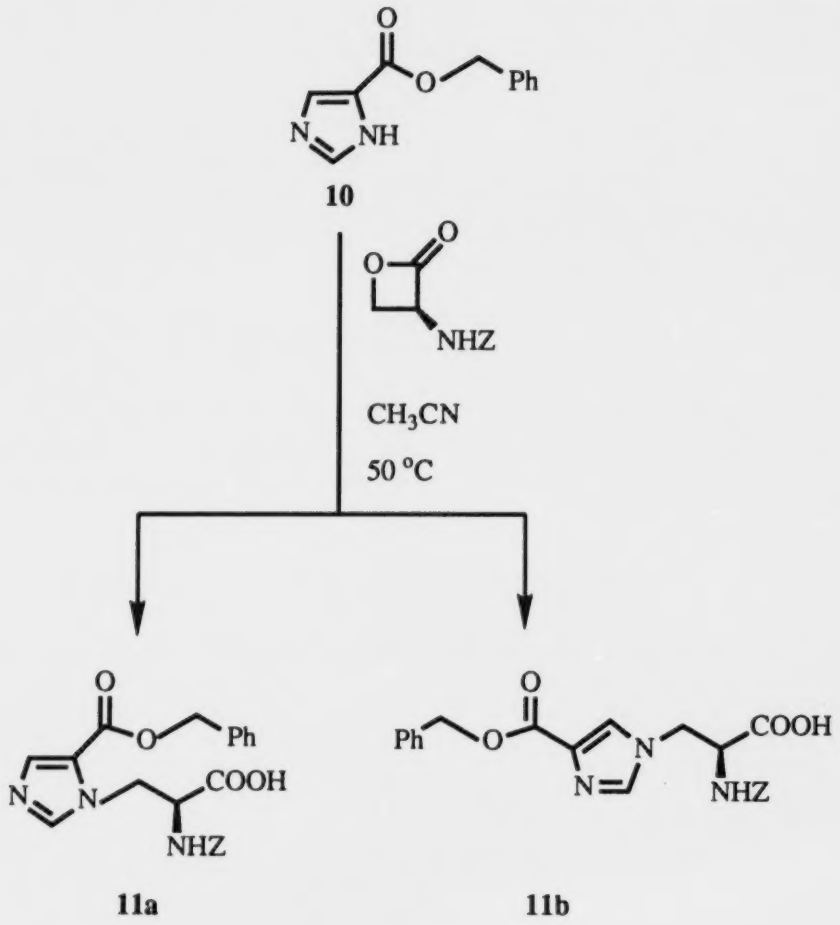
Z= Benzyloxycarbonyl (CBz)

Synthesis of the required nucleophile for preparation of DAP analogue **5** began with commercially available imidazole 4,5-dicarboxylic acid (**8**). Decarboxylation of **8** in refluxing acetic anhydride according to the procedure of Cohen and coworkers[73] affords imidazole 4-carboxylic acid (**9**) in 43% yield (Scheme I). Reaction of **9** with tetramethylammonium hydroxide and benzyl bromide in DMSO[74] forms benzyl ester **10** in 65% yield. Esterification using *p*-toluene-sulfonic acid and benzyl alcohol in toluene[75] proceeds in extremely poor yield, owing to the insolubility of the starting material under the reaction conditions.



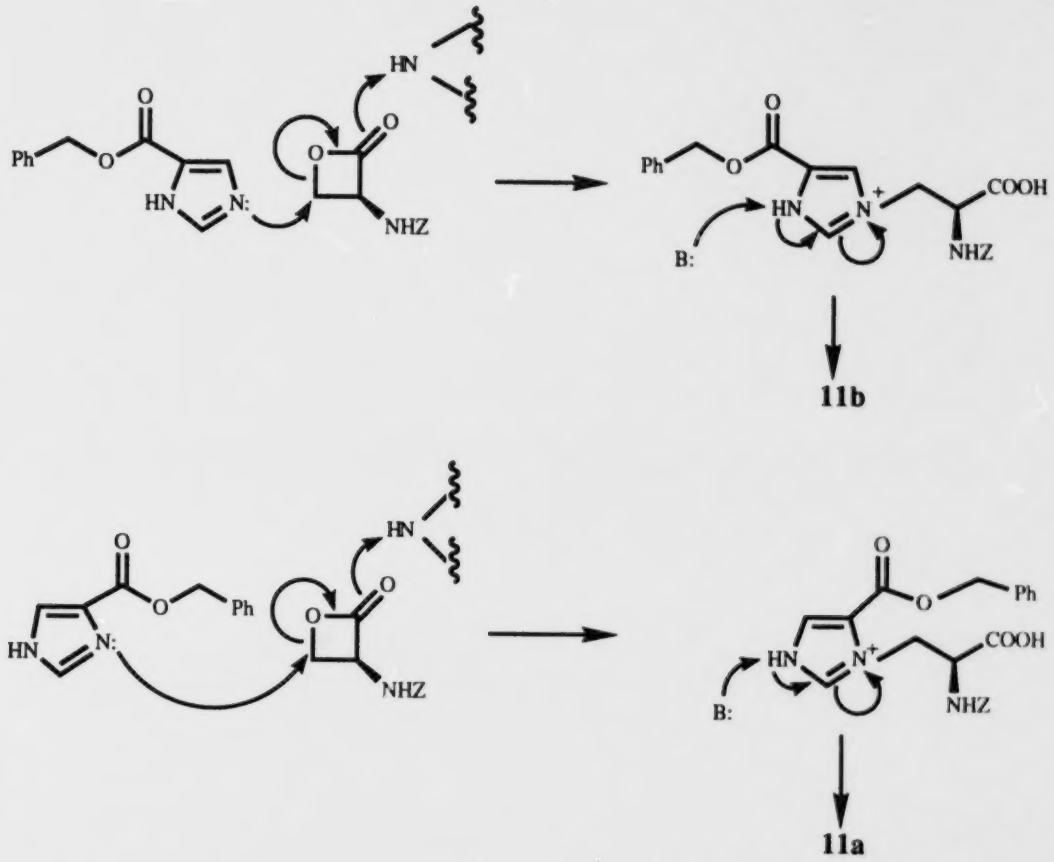
Scheme I

Nucleophilic attack of the benzyl imidazole-4-carboxylate (**10**) on the *N*-benzyloxy carbonyl-L-serine-β-lactone in acetonitrile at 50 °C[67] gives unreacted **10** (34%) and **11a** and **11b** in 13% and 10% yield, respectively (Scheme II). No β-lactone could be recovered from the mixture.



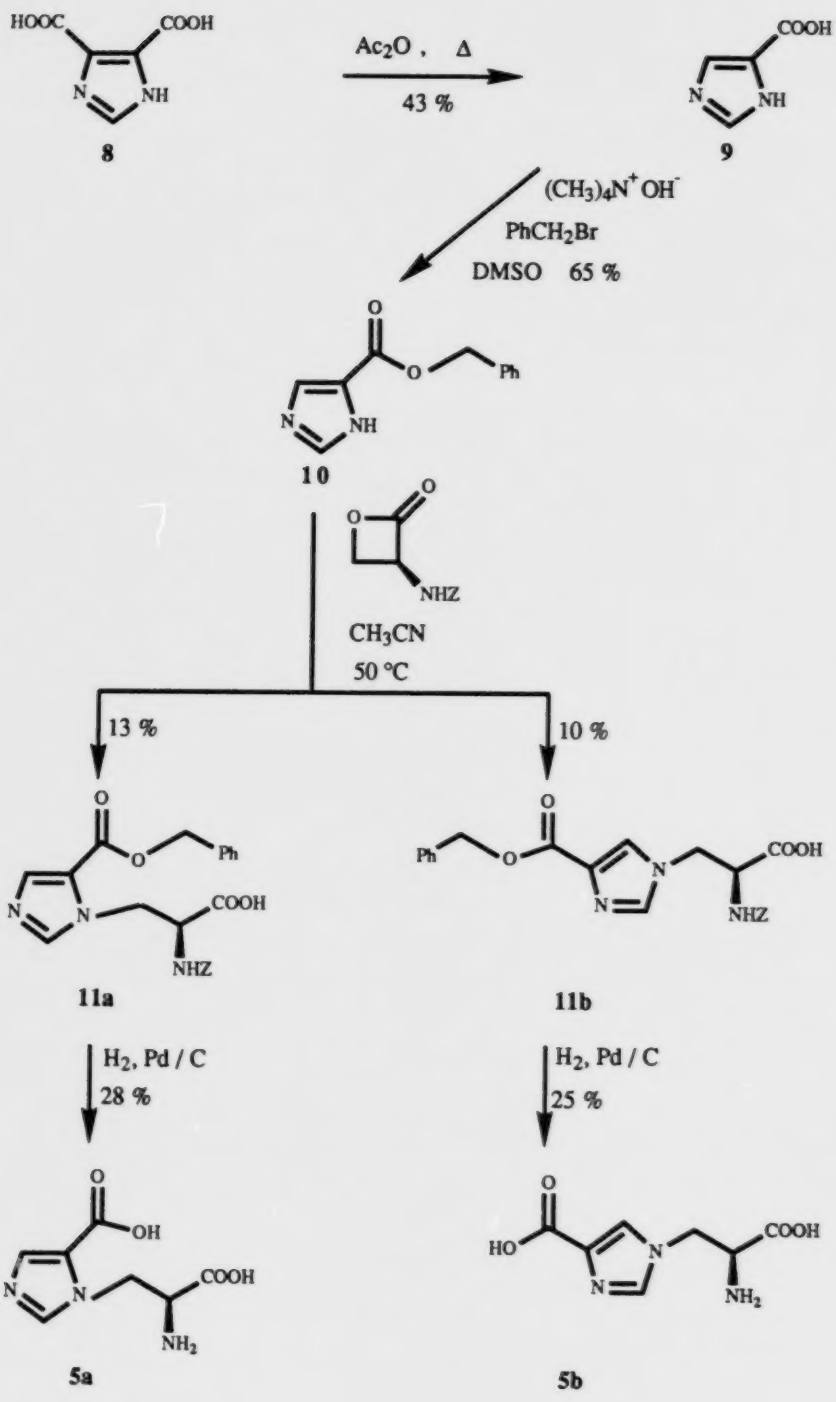
Scheme II

Separation of the two compounds on reverse phase HPLC and spectral analysis showed them to be isomers but could not unambiguously establish their structures. X-Ray crystallographic analysis of **11b** (see appendix) showed it to be the desired imidazole DAP analogue. The formation of the two structural isomers occurs because benzyl ester **10** may have two tautomeric forms, which differ in the position of the imino hydrogen, and can thus attack with either nitrogen,[76] as depicted below.



In order to try to improve the yield of the reaction, the use of bases like NaH and lithium hexamethyldisilazane was investigated but the reactions were unsuccessful. Methyl imidazole-4-carboxylate was also synthesized,[73] using dry CH_3OH and concentrated H_2SO_4 in 37% yield, but its reaction with β-lactone was unsuccessful, probably because of its insolubility in acetonitrile.

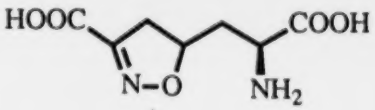
Removal of the benzyl and the benzyloxycarbonyl groups was accomplished by modification of the procedure of Rueppel and coworkers.[77] Hydrogenation of **11a** in methanol yields 28% of the free amino acid **5a**. The same procedure[77] deprotects isomer **11b** to give the corresponding amino acid **5b** in 25% yield (Scheme III).



Scheme III: Schematic representation of the synthesis of imidazole analogue of DAP

2.2 Synthesis of an isoxazoline analogue of DAP

Isoxazolines have been synthesized by a variety of methods including reactions of hydroxylamine with α,β–unsaturated ketones;[78] condensation of alkenes with nitrile oxides;[79] and alkaline degradation of Mannich base methiodides.[80] The strategy for the synthesis of the DAP isoxazoline derivative **4** was based on a 1,3-dipolar cycloaddition reaction of a nitrile oxide with an alkene (Figure 11). This method has been widely used for the synthesis of large number of 2-isoxazolines.[81]



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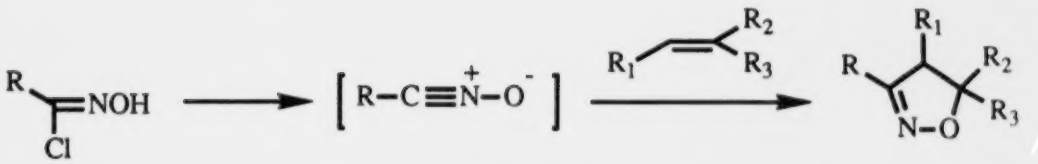
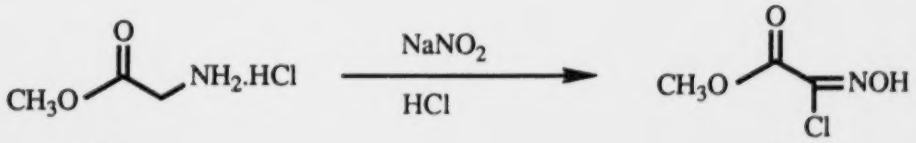


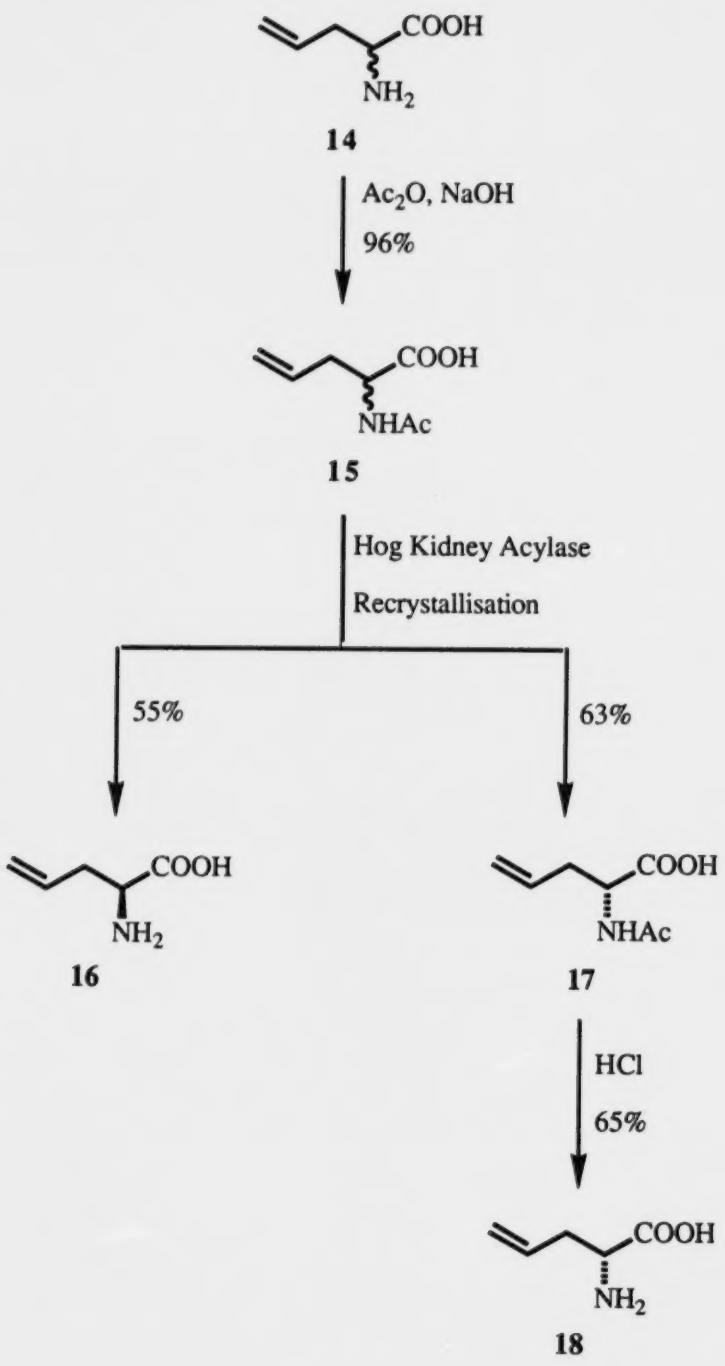
Figure 11: Cycloaddition of a nitrile oxide with an alkene.

The required nitrile oxide can be generated *in situ* from methyl chlorooximidoacetate (**13**), which in turn is available from methyl glycinate hydrochloride (**12**). The procedure of Kozikowski and Adamczyk for preparation of a related compound was followed[79] using stepwise addition of two equivalents each of hydrochloric acid and sodium nitrite at -5 °C to give **13** in 21% yield. (Scheme IV)



12 **13**

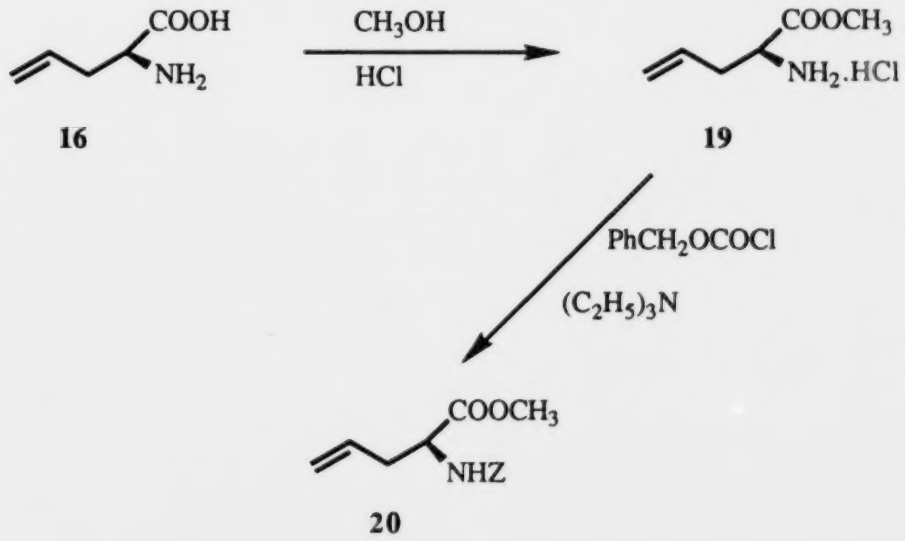
Scheme IV



Scheme V: Resolution of the isomers of 2-amino-4-pentenoic acid from the commercially available racemate.

Synthesis of the dipolarophile, began with protection of the carboxy and amino groups of L-allylglycine, which was obtained by resolution of the commercially available racemate as shown in Scheme V. [82] Optically pure L-allylglycine (99.5% e.e.) was kindly provided by Dr. P. Lane-Bell of our research group. Racemic allylglycine (**14**) was treated with acetic anhydride in aqueous sodium hydroxide to give the acetamide **15**, which on incubation with hog kidney acylase I followed by recrystallization gave the free L-allyl glycine (**16**) in 55% and the D-acetamide **17** in 63% yield. Hydrolysis of **17** in aqueous hydrochloric acid afforded 65% of the free D-allylglycine (**18**) after recrystallization.

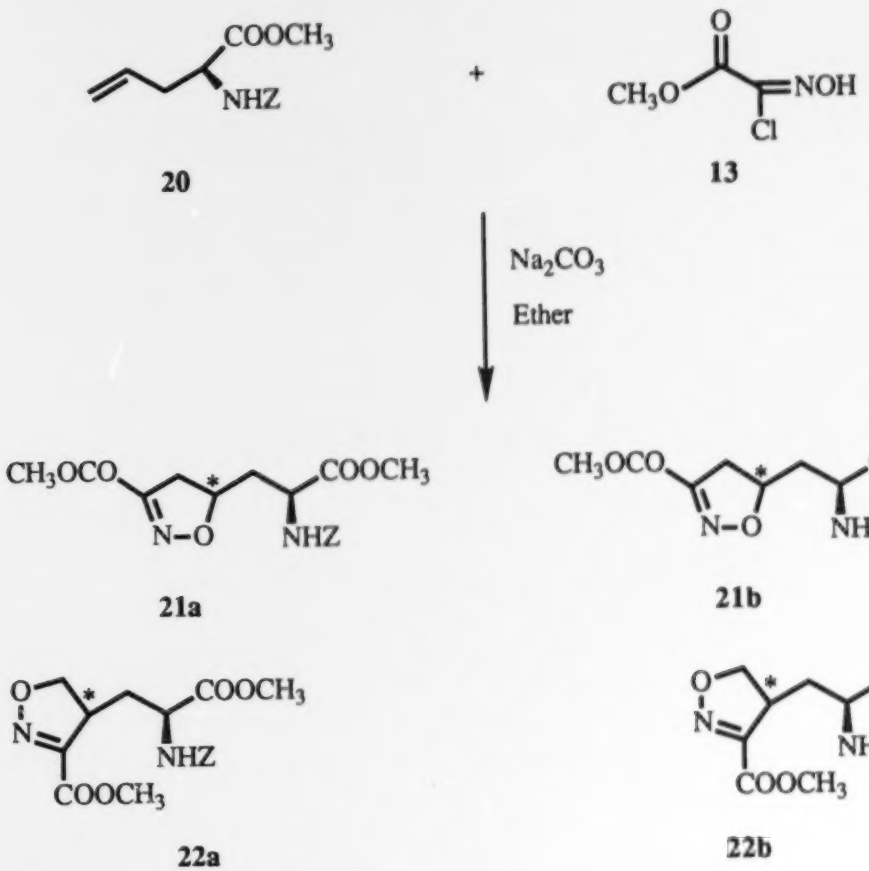
Treatment of optically pure L-allylglycine (**16**) with methanol and hydrogen chloride gas affords 81% of methyl 2-amino-4-pentenoate (**19**).[83] Reaction of **19** with triethylamine and benzyl chloroformate in $CHCl_3$ at -5 °C[84] gives methyl (2S)-2-(*N*-benzyloxycarbonyl) amino-4-pentenoate (**20**) in 55% yield (Scheme VI).



16 **19** **20**

Scheme VI

The 1,3- dipolar cycloaddition was done according to the general procedure of Kozikowski and Adamczyk.[79] Slow addition (3 h) of an aqueous solution of Na_2CO_3 to a vigorously stirred solution of **13** and **20** gives adducts **21a** (41%), **21b** (35%), **22a**(2%) and **22b** (2%) (Scheme VII).



20 **13**

21a **21b**

22a **22b**

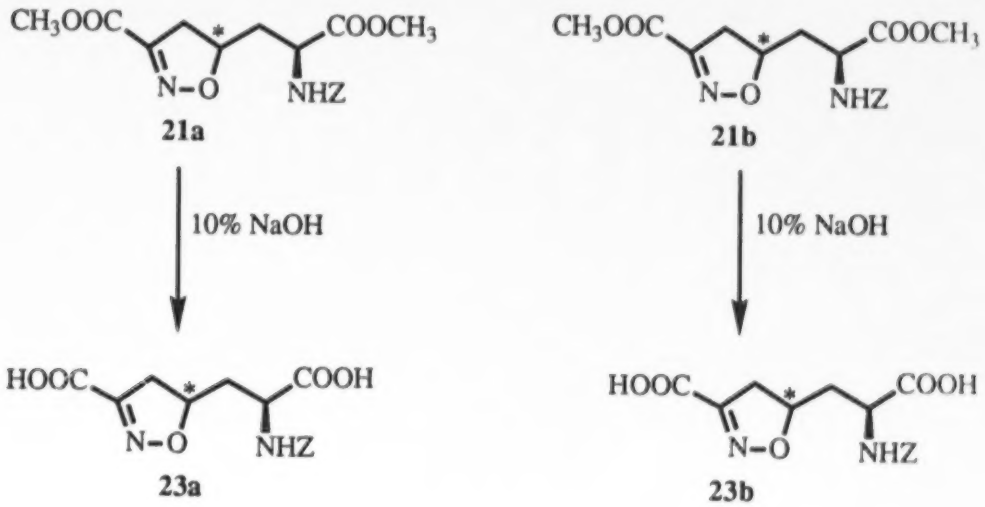
* Single stereoisomer but C-3 stereochemistry unknown

Scheme VII: Dipolar cycloaddition of alkene **20** with the nitrile oxide **13**

Separation of compounds **21a, 21b, 22a** and **22b** by flash chromatography and characterization by ^1H and ^{13}C NMR confirmed their structures. Although chemical studies to determine the ring junction stereochemistry were not completed, crystallization of

the four compounds for X-ray analysis was attempted. Compound **21a** and **21b** were successfully crystallized, but the crystals proved to be unsuitable for X-ray crystallography.

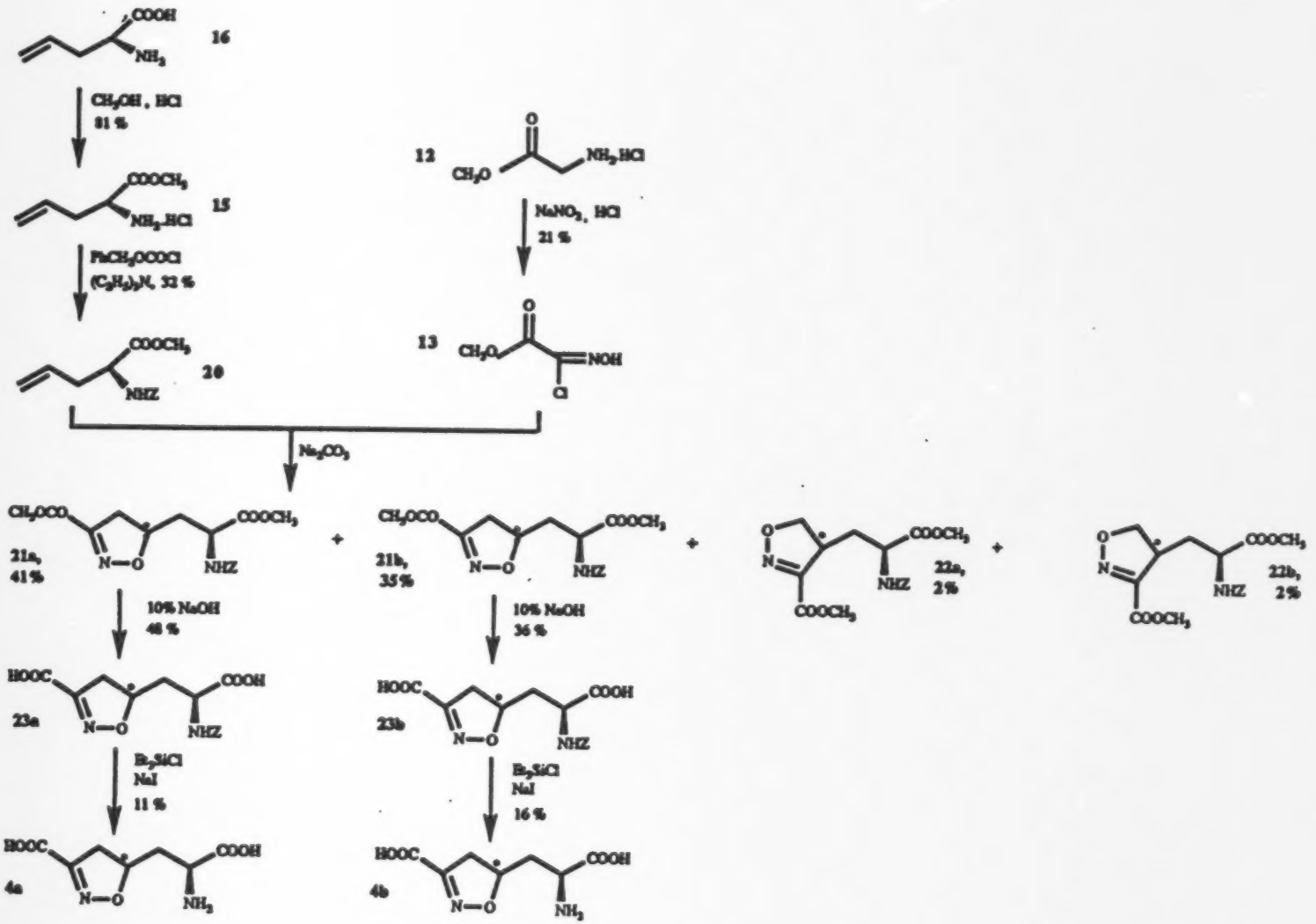
Hydrolysis of the methyl esters **21a** and **21b** employed the procedure of Vaughan and Spencer.[85] Thus cleavage of **21a** with 10% sodium hydroxide followed by purification on reverse phase HPLC gave 48% of the dicarboxylic acid **23a**. A similar reaction deprotected **21b** to give **23b** in 36% yield (Scheme VIII).



* Single stereoisomer but C-3 stereochemistry unknown

Scheme VIII: Hydrolysis of the cycloadducts **21a** and **22b**

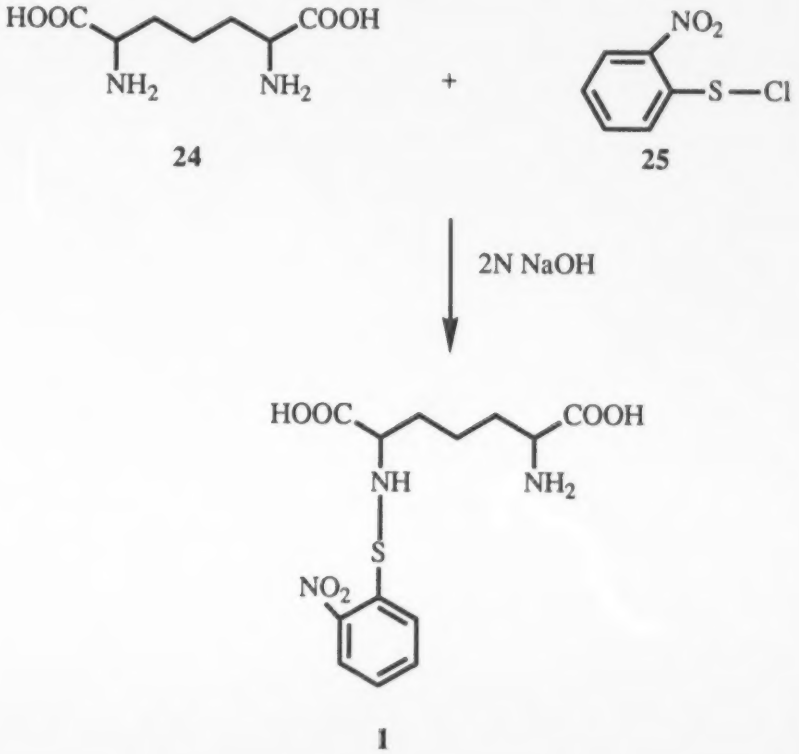
Removal of the benzyloxycarbonyl (Cbz) group of **23a** with sodium iodide and chlorotrimethylsilane [86] gave, after purification by reverse phase HPLC, 11% of the pure amino acid **4a**. Analysis of the reaction mixture by TLC did not show any starting material and the low recovery is attributed to purification problems. The same procedure[86] also deprotected **23b** to give 16% yield of the amino acid **4b** (Scheme IX).



Scheme IX: Schematic representation of the synthesis of isoxazoline analogue of DAP

2.3 Synthesis of sulfur analogues of DAP

Syntheses of the sulfenamide, sulfinamide and sulfonamide analogues of DAP were based on the use of the *o*-nitrophenylsulfenyl group (NPS) as an *N*-protecting group during coupling reaction in peptide synthesis. The procedure of Gordon and coworkers[87] for the preparation of related compounds was adapted. Commercially available DAP **24** (statistical mixture of all possible stereoisomers) was treated with sodium hydroxide and *o*-nitrophenylsulfenyl chloride **(25)** in deionized H_2O and THF to give **1** in 15% yield after reverse phase HPLC purification (Scheme X).



24 **25** **1**

Scheme X

Unreacted DAP and *o*-nitrophenyl sulfenyl chloride were detected in the aqueous and organic layers respectively. Increase in the concentration of sodium hydroxide did not seem to affect the outcome of the reaction.

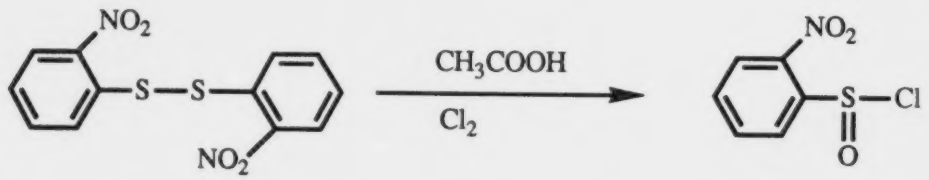
For the synthesis of 2-(*N*-*o*-nitrophenylsulfinyl) diaminopimelic acid **(2)**, the preparation of *o*-nitrophenylsulfinyl chloride compound **(27)** was necessary. A procedure involving chlorination of disulfides for the preparation of sulfinyl chlorides has been developed by Douglass and coworkers.[88] Stoichiometry of the reactants seems to be critical for the success of the reaction based on its proposed mechanism:

$$ArSSAr + Cl_2 \longrightarrow 2\,ArSCl$$

$$ArSCl + Cl_2 \longrightarrow ArSCl_3$$

$$ArSCl_3 + CH_3COOH \longrightarrow ArSOCl + CH_3COCl + HCl$$

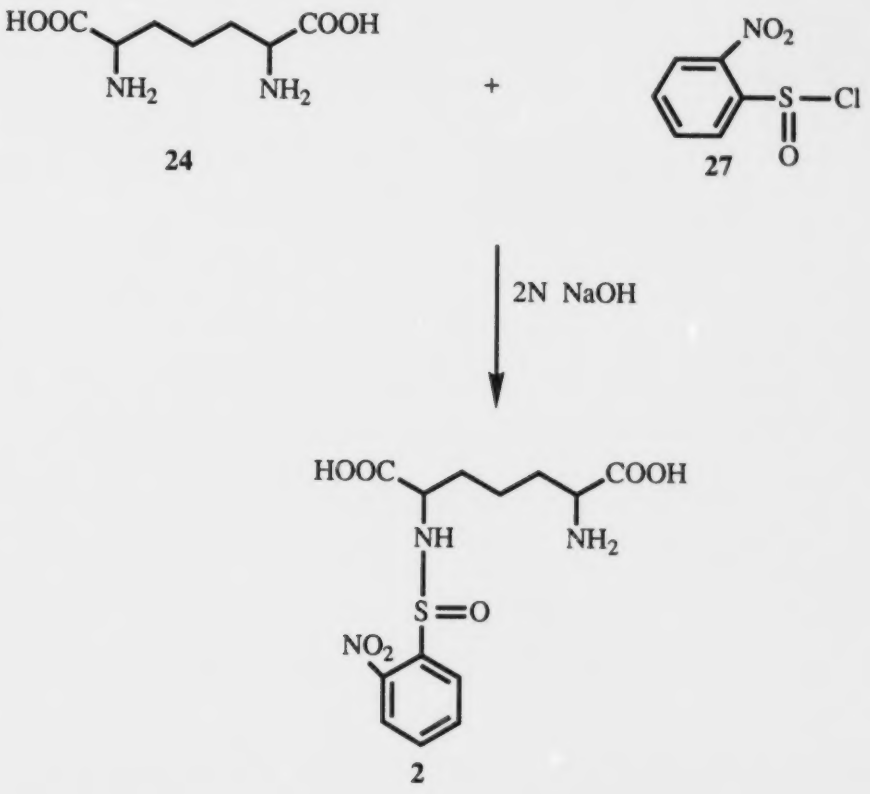
Sulfenyl chloride and sulfonyl chloride are common contaminants resulting from failure to maintain the correct stoichiometry of the reactants. The procedure of Oae and Ikura[89] for the preparation of related compounds was adapted. Bis-(*o*-nitrophenyl)disulphide **(26)** was treated with two equivalents of glacial acetic acid followed by chlorine gas at -5 °C to give a 58% yield of **27** (Scheme XI).



26 **27**

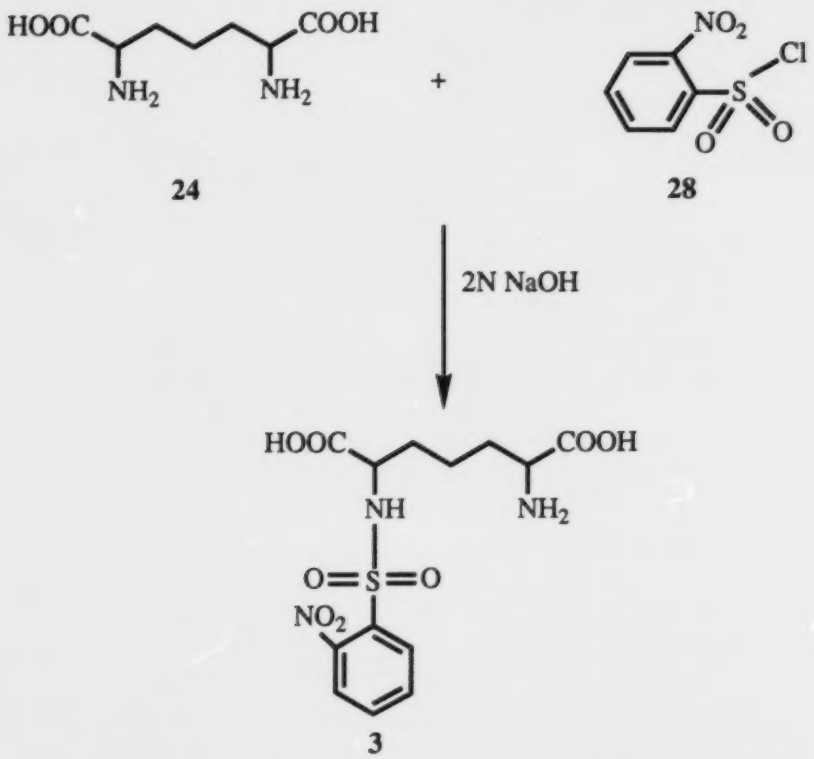
Scheme XI

The procedure of Gordon and coworkers for the preparation of *N*-sulfenyl amino acids, was adapted [87] for preparation of **2**. Commercially available DAP **24** (statistical mixture of all possible isomers) was treated with aqueous sodium hydroxide and *o*-nitrophenyl sulfinyl chloride (**27**) in THF to give, after HPLC purification, compound **2** in 9% yield (Scheme XII).



Scheme XII

A similar approach was used for the synthesis of 2-(*N*-*o*-nitrophenylsulfonyl) diaminopimelic acid **3**. Aqueous sodium hydroxide was added to DAP **24** (statistical mixture of all possible isomers), with simultaneous addition of *o*-nitrophenylsulfonyl



Scheme XIII

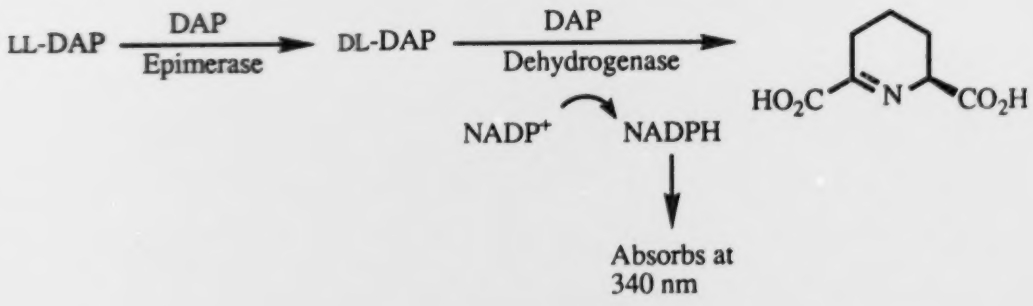
chloride (**28**) in THF to afford, after reverse phase HPLC, a 16% yield of **3** (Scheme XIII).

2.4 Isolation of DAP epimerase and inhibition studies

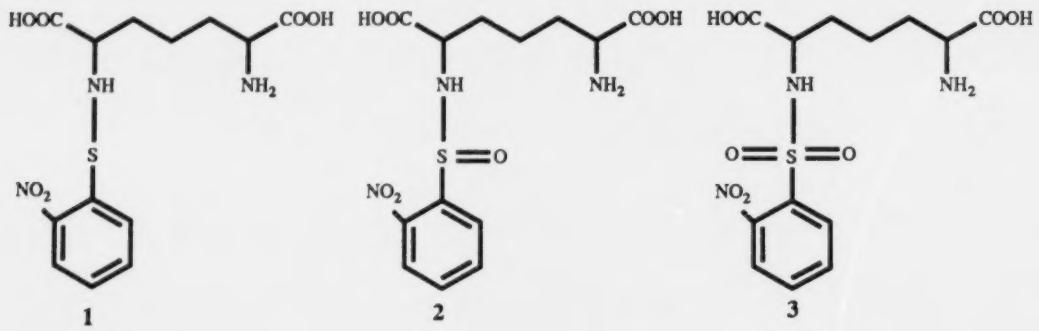
With the successful completion of the synthesis of the DAP analogues **1-5**, attention was focussed on testing of these compounds as potential inhibitors of DAP epimerase. This required the isolation and purification of this enzyme, and the procedure of Higgins and coworkers was adapted.[35] Recombinant *Escherichia coli* LE134, which is an overproducer of DAP epimerase, was kindly grown by Dr. Michael Pickard (Department of Microbiology, University of Alberta), who also prepared an extract by sonication of the

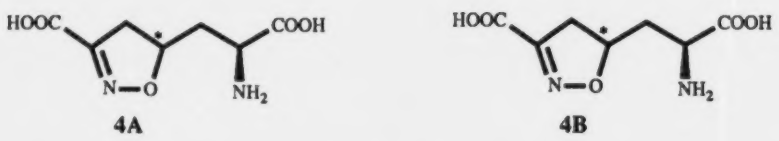
cells and centrifugation to remove debris. The extract was subjected to ammonium sulphate fractionations, followed by a DE-52 cellulose and Matrix™ Gel Green A column chromatography. Assay of the fractions, dialysis and ultrafiltration of the active fractions gave two enzyme solutions with specific activities of 79 and 32 U/mg respectively, where one unit of activity is defined as one μmol/min of NADPH formed under the conditions of the coupled enzyme assay (see below). Bio-Rad Mini-protean II Ready Gel (12% polyacrylamide) was used to confirm the purity of the enzyme (Figure 12, page 54) which showed a single major band, corresponding to the epimerase, accompanied by 2-3 minor bands. The final enzyme solutions were stored in the buffer containing 10 mM DTT and were tightly capped as studies indicated that the thiol group present in the active site of the enzyme could be readily oxidized resulting in loss of enzymatic activity.[50]

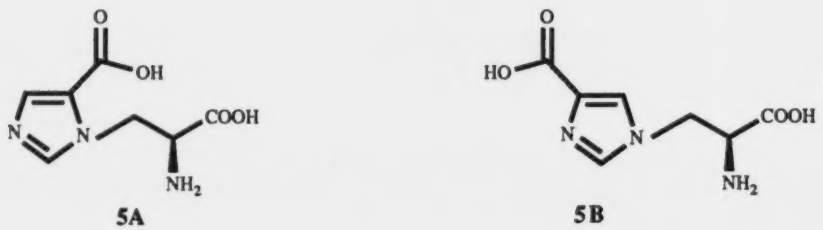
With the pure enzyme available, inhibition studies with the potential inhibitors were initiated. A coupled enzyme assay allows monitoring of the DAP epimerase activity.[46] The assay relies on the conversion of pure LL-DAP to *meso*-DAP. The latter can be detected using excess DAP dehydrogenase (obtained as a gift from Ms. Suzanne Crawley, Dept. of Food Science, University of Alberta), which simultaneously converts $NADP^+$ to NADPH. Spectroscopic monitoring of this process at 340 nm provides the means for detecting the conversion. The required LL-DAP was obtained by resolution from a mixture of all isomers of diaminopimelic acid[90] by Dr. Lee Arnold from our research group.



The compounds were first tested for their ability to act as substrates or inhibitors of DAP dehydrogenase to eliminate the possibility of interference with the coupled enzyme assay. These experiments were done using 1.0 mM concentration of the analogue and following any increase in the absorbance at 340 nm. None of the compounds **1**, **2**, **3**, **4a**, **4b**, **5a**, **5b** were substrates of DAP dehydrogenase.







One point tests of the compounds as a competitive inhibitors of DAP dehydrogenase employed 1.0 mM concentration of *meso*-DAP as substrate, 1.8 mU DAP dehydrogenase and 1.0 mM concentration of the inhibitor. Control and inhibition runs showed that compounds **1**, **2**, **3**, **4a**, **5a** and **5b** were not significant inhibitors of dehydrogenase (concentration for 50% inhibition > 1 mM). However, isoxazoline **4b** proved to be a good

competitive inhibitor of DAP dehydrogenase, showing 50% inhibition at 20 μM concentration of the inhibitor.

Since all of the compounds except **4b** were neither substrates nor inhibitors of DAP dehydrogenase, the coupled enzyme assay could be used to study their effects on DAP epimerase. Tests were done using 0.4 mM concentration of the substrate (LL-DAP) and 1.0 mM concentration of the inhibitor. DAP sulfenamide **1**, sulfinamide **2** and sulfonamide **3** were found to be very weak inhibitors showing 5%, 7% and 5% inhibition respectively. Increase in the inhibitor concentration (3.0 mM) did not seem to have any substantial effect on the percentage inhibition of DAP sulfenamide (15%) or sulfonamide (11%) although a slight increase was observed for the sulfinamide analogue (38%). The isoxazoline analogue **4a** also proved to be a very weak inhibitor of epimerase showing only 18% inhibition at 1.0 mM concentration of the inhibitor. The imidazole analogues **5a** and **5b** were also weak and showed 11% and 18% inhibition, at 1.0 mM concentration of the analogues respectively.

Since compound **4b** is an inhibitor of DAP dehydrogenase, the coupled enzyme assay could not be used to study its effects on DAP epimerase. Hence, a radioactive assay [46] was employed which is based on the incubation of tritiated [^{3}H]-DAP directly with the epimerase. Subsequent elution on a cation exchange column allows determination of the tritium [^{3}H] released. Inclusion of the inhibitor in an analogous run and comparison with the control gives the percentage inhibition. Compound **4b** interacts only weakly with DAP epimerase and shows only 4% inhibition at 0.5 mM concentration of the inhibitor.

All the analogues **1**, **2**, **3**, **4a**, **4b**, **5a** and **5b** were tested for time-dependent inactivation of the epimerase using the coupled enzyme assay. However no time dependent inhibition

Table I. Inhibition of DAP enzymes by substrate and transition state analogues.

Analogues	DAP Dehydrogenase	DAP Epimerase
1	9% (1.0 mM)	5% (1.0 mM) [a]
2	6% (1.0mM)	7% (1.0 mM) [a]
3	7% (1.0 mM)	5% (1.0 mM) [a]
4a	13% (1.0 mM)	18% (1.0 mM) [a]
4b	50% (20 μM)	4% (0.5 mM) [b]
5a	4% (1.0 mM)	11% (1.0 mM) [a]
5b	7% (1.0 mM)	18% (1.0 mM) [a]

[a] examined by coupled enzyme assay.

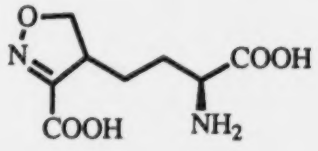
[b] tritium release assay.

could be observed for any of the compounds for incubations of up to 60 min with an inhibitor concentration of 1.0 mM.

2.5 Conclusion

Although the enzyme mechanism and substrate specificities stimulated the design of the DAP analogues **1-5**, the weak inhibitory properties of these compounds indicate that more information is needed about of the mechanism and possible intermediates. The functional groups clearly play an important role in enzyme recognition because both amino and carboxyl groups must be present for DAP epimerase and DAP dehydrogenase.[48,50]

The failure of DAP sulfenamide **1**, sulfinamide **2** and sulfonamide **3** to bind to these enzymes may be due to steric bulk or the lack of basicity at the nitrogen bearing these groups. Initial hopes that such sulfur containing groups could be eliminated by the epimerase, as occurs with the *N*-hydroxy and β–fluoro DAP's, could not be realized because of this lack of recognition by the enzyme. More disappointing is the failure of imidazole analogue **5b** and one of the isoxazoline isomers **4a** or **4b** to act as transition state analogues for the epimerase. These compounds possess two basic nitrogens, two carboxyl groups, and approximately the correct chain length required for binding. The additional atoms and constrained geometry of these analogues may permit correct docking on the active site surface of the epimerase. Design of these materials was based on a fully extended DAP conformation wherein the carboxyl groups are maximally separated. This concept produced a reasonably good competitive inhibitor of the dehydrogenase, namely **4b**. However, it may be that a rotomer having one amino group and the distal carboxyl at maximum distance better approximates the preferred substrate geometry for the epimerase. Synthesis of the corresponding isoxazoline analogues (see below) is currently being investigated in our laboratory.



If successful, such rigid analogues would not only provide insight into the active site geometry and mechanism of the epimerase but would also generate useful broad spectrum antibiotics.

3. EXPERIMENTAL

3.1 General

All the reactions involving air sensitive reagents were done under a slight positive pressure of dry argon. Dry reactions were performed using oven dried glassware (~ 12h at 125-140 °C) which were cooled under argon before the start of the reaction. Milli Q (Millipore) H_2O was used for reactions and was degassed for HPLC. All the solvents were dried under argon according to Perrin and Armarego.[91] Tetrahydrofuran (THF) was distilled from Na or K and benzophenone. Acetonitrile (CH_3CN) and triethylamine (Et_3N) were distilled over calcium hydride (CaH_2). Methanol was distilled over Mg with catalytic I_2. Dimethyl sulphoxide (DMSO) was dried over Linde Type 4A molecular sieves and fractionally distilled under reduced pressure. The solid reactants were dried *in vacuo* using a vacuum pump for 6-8 h. All organic layers obtained from extractions were dried over Na_2SO_4. All solvents used for chromatography were distilled. All the reagents used were of ACS grade.

The progress of reactions was monitored on thin layer chromatography (TLC) using one or more of the following visualizing agents: UV light by fluorescence quenching; I_2 staining; ninhydrin (for amino acids); phosphomolybdic acid; bromocresol green (for carboxylic acids). Commercially available thin layer plates used were: normal silica, Merck 60 F-254; reverse phase, Merck RP-8 F-254S. Flash chromatography was performed according to the method of Still [92] under argon pressure using silica gel Merck type 60, 230-420 mesh. All the solvent mixtures listed are volume ratios. High pressure liquid chromatography was performed on BioRad Gradient Module with two BioRad Model 1350 HPLC pumps

and a Rheodyne injector, using a BioRad UV Monitor model 1305A detector and control unit, controlled by a Model 400 Chromatography Data Station running BioRad GPS Software. Reverse phase columns used on HPLC were Waters Millipore Radial Compression module (10 cm x 8 mm) with Radial-Pak C_{18} cartridge. Analyses of amino acids was done on a Waters Millipore Radial Compression module (10 cm x 8 mm) with a Microbonda Pak aminopropyl cartridge.

Melting points were determined on a Büchi oil immersion apparatus using open capillary tubes. All literature compounds had ^{1}H NMR, IR and MS consistent with the assigned structures. ^{1}H NMR and ^{13}C NMR spectra were acquired on Bruker WP-80, WH-200, WH-400 instruments. IR spectra were acquired on a Nicolet Analytical Instruments 20SX instrument. Mass spectra were done on an AEI MS12 (Chemical ionisation) and AEI MS9 (POS FAB) instruments. Cleland matrix (composed of dithiothreitol / dithioerythritol in a 6:1 ratio) was used for POS FAB spectra. The NMR samples were analyzed in deuterated solvents containing internal references such as tetramethylsilane (TMS) in organic solvents or sodium 3-(trimethyl silyl)-1-propanesulphonate (TSP) in D_2O. Deuterated solvents were used for reference in ^{13}C NMR spectroscopy.

For the enzymatic work, dithiothreitol (DTT) was purchased from Diagnostic Chemicals Ltd. (West Royalty Industrial Park, Charlettown, P.E.I., Canada) and Sigma. $NADP^{+}$ and bovine serum albumin were obtained from Sigma. DEAE cellulose was obtained from Whatman, and Matrix™ Green A from Amicon (Beverly, Massachusetts, U.S.A.). Spectropor dialysis tubing size 4 (molecular weight cut off 12 000-14 000), Tris and Scintiverse E scintillation cocktails were obtained from Fisher Scientific. [G-^{3}H] DAP (statistical mixture of isomers 1.09 Ci/mmol) was purchased from Amersham (Arlington Height, Illinois, U.S.A.). All purification steps in the isolation of the enzymes were done in a cold room at 4 °C. Centrifugation was performed on a Sorvall RC-5B Refrigerated

Superspeed centrifuge using the GSA rotor at 4 °C. Spectrophotometric assay of the supernatants and the pellets were done on a Hewlett-Packard 8452A Diode Array Spectrophotometer. Ultrafiltration employed Diaflo Ultrafilter PM10 membranes (Cut off 10 000 kDa). Electrophoresis was performed using a BioRad 3000Xi computer controlled power supply and a BioRad MiniProtean vertical slab unit. Acrylamide gel reagents were purchased from BioRad. Radioactivity was determined on a Beckman LS5000TD scintillation counter.

3.2 Synthesis

2-(*N*-*o*-Nitrophenylsulfenyl)diaminopimelic Acid (1)

The procedure of Gordon and coworkers for the preparation of related compounds was adapted.[87] To a stirred suspension of diaminopimelic acid **24** (statistical mixture of isomers) (0.100 g, 0.525 mmol) in 5 mL of deionized H_2O was added simultaneously 2N NaOH (1.97 mL) and a solution of *o*-nitrophenylsulfenyl chloride **25** (0.109 g, 0.576 mmol) in 5 mL of THF over a period of 10 min. The reaction mixture was stirred for 30 min and deionized H_2O (10 mL) was added to it. A 10% solution of $KHSO_4$ was used to acidify the mixture to pH 2.5, which was then washed with ethyl acetate (3 x 20 mL). The aqueous layer was adjusted to pH 7 and then lyophilised. Purification on reverse phase HPLC (C18 column) gave 26 mg (15%) of **1**: IR (KBr): 3400, 2920, 1630, 1593, 1503, 1407, 1338, 1306, 1056 cm^{-1}; ^1H NMR (200 MHz, D_2O) δ 8.22-8.15 (m, 1H, Ar*H*), 7.90-7.83 (m, 1H, Ar*H*), 7.71-7.60 (m, 1H, Ar*H*), 3.82-3.72 (m, 1H, C*H*NS), 3.60-3.48 (m, 1H, C*H*NH_2), 1.90-1.71 (m, 4H, 2 x CH_2CN), 1.63-1.40 (m, 2H, CH_2C); MS (POS FAB): 344 (MH^+).

2-(*N*-*o*-Nitrophenylsulfinyl)diaminopimelic Acid (2)

The procedure of Gordon and coworkers for the preparation of related compounds was adapted.[87] To a stirred suspension of diaminopimelic acid **24** (statistical mixture of isomers) (0.100 g, 0.525 mmol) in deionized H_2O (3 mL) were added simultaneously 2N NaOH and *o*-nitrophenylsulfinyl chloride **(27)** (0.118 g, 0.576 mmol) in THF (3 mL) over 10 min. The reaction mixture was stirred for 30 min and deionized H_2O (6 mL) was added to it . The reaction mixture was acidified to pH 2.5 using 10% $KHSO_4$ solution and was washed with ethyl acetate (3 x 10 mL). The aqueous layer was adjusted to pH 7 and then lyophilised. Purification on reverse phase HPLC (C18 column) gave 15 mg (9%) of **2**: IR (KBr) 3400 (br), 2910, 1655, 1590, 1505, 1405, 1338, 1151 ; ^{1}H NMR (200 MHz, D_2O) δ 8.40-8.28 (m, 1H, Ar*H*), 8.04-7.95 (m, 1H, Ar*H*), 7.85–7.73 (m, 1H, Ar*H*), 7.48-7.32 (m, 1H, Ar*H*), 3.87-3.70 (t, 1H, C*H*NS), 3.48-3.30 (m, 1H, C*H*NH_2), 2.01-1.72 (m, 4H, 2 x CH_2CH), 1.69-1.43 (m, 2H, CH_2C); MS (POS FAB) 382 $(M+ Na)^+$, 404 $(M+2Na)^+$.

2-(*N*-*o*-Nitrophenylsulfonyl)diaminopimelic Acid (3)

The procedure of Gordon and coworkers for the preparation of related compounds was adapted.[87] To a stirred suspension of diaminopimelic acid **24** (statistical mixture of isomers) (0.100 g, 0.525 mmol) in deionized H_2O (5 mL) was added simultaneously 2N NaOH (1.19 mL) and a solution of *o*-nitrophenylsulfonyl chloride **(28)** (0.216 g, 0.576 mmol) in 5 mL of THF over a period of 10 min. The reaction mixture was stirred for 30 min and deionized H_2O (10 mL)was added to it. The reaction mixture was acidified with 10% $KHSO_4$ to pH 2.5 and was washed with ethyl acetate (3 x 20 mL). The aqueous layer was adjusted to pH 7 and then lyophilised. Purification on reverse phase HPLC (C18 column) gave 30 mg (16%) of **3**: IR (KBr) 3438 (s, br), 3100, 2930, 1630, 1541, 1330, 1138 cm^{-1}; ^{1}H NMR (200 MHz, D_2O) δ 8.18-8.07 (m, 1H, Ar*H*), 8.03-7.97 (m, 1H, Ar*H*), 7.94-7.81 (m, 2H, Ar*H*), 4.14-4.01 (q, 1H, *J* =4.0 Hz, C*H*NH), 3.90-3.78 (t, 1H,

J =6.0 Hz, C*H*NH_2), 2.01-1.71 (m, 4H, 2 x CHCH_2), 1.63-1.40 (m, 2H, $CH_2CH_2CH_2$); MS (POS-FAB, Cleland) 376 (MH^+).

(2*S*)-2-Amino-3-(3-carboxy-2-isoxazolin-5-yl)propionic Acid (4a)

The procedure of Olah and coworkers for the preparation of related compounds was adapted.[86] To a solution of **23a** (0.060 g, 0.178 mmol) and sodium iodide (0.080 g, 0.535 mmol) in dry acetonitrile (3 mL) was added chlorotrimethylsilane (0.045 mL, 0.357 mmol) over a period of 5 min. The yellow solution was stirred for 1 h and then treated with deionized H_2O (8 mL). The solution was then washed with ether (3 x 15 mL) and the aqueous layer was freeze dried. Purification by reverse phase chromatography (aminopropyl column) gave 4 mg (11%) of **4a**: IR (neat film) 3440, 1680, 1650, 1440, 1410, 1210, 1140 cm^{-1}; ^1H NMR (200 MHz, D_2O) δ 5.10-5.01 (m, 1H, C*H*O), 3.92 (dd, 1H, *J* =5.0, 8.0 Hz, C*H*N), 3.44 (dd, 1H, *J* =10.0, 17.5 Hz, C*H*C=N), 2.96 (dd, 1H, *J*=11.0, 17.5 Hz, C*H*C=N), 2.32-2.03 (m, 2H, CH_2CHN); MS-CI 203 (MH^+).

(2*S*)-2-Amino-3-(3-carboxy-2-isoxazolin-5-yl)propionic Acid (4b)

The procedure of Olah and coworkers for the preparation of related compounds was adapted.[86] To a solution of **23b** (0.034 g, 0.101 mmol) and sodium iodide (0.030 g, 0.201 mmol) in dry acetonitrile (3 mL) was added with continuous stirring chloro-trimethylsilane (0.026 mL, 0.202 mmol) over a period of 5 min. The yellow solution was stirred for 1 h and deionized H_2O (5 mL) was added. This solution was then washed with ether (3 x 10 mL) and the resulting aqueous layer was freeze dried. The crude extract was purified by reverse phase chromatography (aminopropyl column) to give 4 mg (16%) of isomer **4b**: IR (KBr) 3440(s, br), 2920, 1648, 1605, 1305, 1160 cm^{-1}; ^1H NMR (200 MHz, D_2O) δ 5.02-4.85 (m, 1H, C*H*O), 3.97 (q, 1H, *J*=5.0 Hz, C*H*N), 3.44 (dd, 1H, *J*=10.0, 18.0 Hz, C*H*C=N), 3.05-2.90 (dd, 1H, *J* =8.0, 18.0 Hz, C*H*C=N), 2.29-2.18 (m, 2H, CH_2CHN); MS-CI 203 (MH^+).

(2*S*)-2-Amino-3-(5-carboxy-1-imidazolyl)propionic Acid (5a)

The procedure of Rueppel *et al.*[77] was modified. A mixture of **11a** (0.047 g, 0.110 mmol) in MeOH (5 mL) and 10% (w/w) of Pd on carbon (47 mg) was stirred under H_2 (1 atm) at room temperature for 2 h. The resulting mixture was filtered and the filtrate was concentrated *in vacuo* to give an oil. This was purified on a reverse phase HPLC (C18 column) using 25% acetonitrile and 75% H_2O to give 6 mg (28%) of **5a**: IR (MeOH cast) 3400 (s, br), 2985, 1675, 1640, 1428, 1390, 1210, 1140 cm^{-1}; ^1H NMR (200 MHz, D_2O) δ 8.82 (s, 1H, C*H*=C), 7.91 (s, 1H, C*H*=N), 5.19 (dd, 1H, *J* =4.0, 14.0 Hz, CH_2CH), 4.98 (dd, 1H, *J* =4.0, 14.0 Hz, CH_2CH), 4.33 (t, 1H, *J* =4.0 Hz, C*H*CH_2); MS (POS FAB, HCOOH /Glycerol) 200 (MH^+).

(2*S*)-2-Amino-3-(4-carboxy-1-imidazolyl)propionic Acid (5b)

The procedure of Rueppel *et al.*[77] was modified. A mixture of **11b** (0.032 g, 0.075 mmol) in MeOH (5 mL) and 10% (w/w) of Pd on carbon (32 mg) was stirred under H_2 (1 atm) at room temperature for 2 h. The resulting mixture was filtered and the filtrate was concentrated *in vacuo* to give an oil. This was purified on a reverse phase HPLC (C18 column) using 25% acetonitrile and 75% H_2O to give 3.80 mg (25%) of **5b**: IR (MeOH cast) 3400 (s, br), 2980, 1677, 1639, 1428, 1390, 1205, 1139 cm^{-1}; ^1H NMR (200 MHz, D_2O) δ 8.82 (s, 1H, C*H*=C), 7.95 (s, 1H, C*H*=N), 5.20-4.90 (m, 2H, CH_2CH), 4.35 (t, 1H, *J* =4.0 Hz, C*H* CH_2); MS (POS FAB, HCOOH /Glycerol) 200 (MH^+)

4-Imidazole Carboxylic Acid (9)

The procedure of Cohen and coworkers was followed.[73] A suspension of imidazole 4,5-dicarboxylic acid **(8)** (1.02 g, 6.40 mmol) in 37.5 mL of acetic anhydride was refluxed for 5 h, at which point the solution was complete. The solution was filtered in order to remove some precipitate and the filtrate was concentrated *in vacuo* at 60 °C to a dark brown viscous residue. To the residue was added deionized H_2O (15 mL) and the

mixture was stirred overnight, to give a cream coloured precipitate. The precipitate dissolved on heating of the mixture on a steam bath for 1 h. To the resulting brown solution was added ethanol (15 mL) and decolourizing charcoal (Norit A). The mixture was heated on steam bath for 30 min. This was filtered hot and refrigerated overnight to give a white precipitate. The precipitate was filtered and dried to give 0.309 g (43%) of pure **9**: mp 270-272 °C (dec.), lit.[93] mp 271 °C (dec.); IR (KBr) 3640-33310 (s, br), 2991, 1621, 1446, 1373, 910, 858, 536 cm^{-1}; ^1H NMR (200 MHz D_2O) δ 8.68 (s, 1H, C*H*=C), 7.75 (s, 1H, C*H*=N); CI-MS 113 (MH^+) ; Anal. Calcd. for $C_4H_4N_2O_2$: C, 42.85; H, 3.59; N, 24.99%. Found: C, 42.74; H, 3.60; N, 24.96%.

Benzyl Imidazole-4-Carboxylate (10)

The procedure of Wagenknecht and coworkers for the preparation of related compounds was adapted.[74] To dry DMSO (5 mL) was added 4-imidazole carboxylic acid (**9**) (0.100 g, 0.892 mmol) and tetramethyl ammonium hydroxide (0.149 g, 0.821 mmol). To this solution was added benzyl bromide (0.098 mL, 0.825 mmol) and the solution was stirred overnight. Precipitation was observed as the reaction proceeded. The reaction mixture was then added to deionized H_2O (10 mL) and this was extracted with diethyl ether (3 x10 mL). The crude mixture was purified by silica gel flash chromatography (EtOAc/ formic acid, 97/3) to give 0.108 g (65%) of pure **10**: mp 159-163 °C; IR ($CHCl_3$ cast) 3600-3320 (m, br), 3015, 2980, 1723, 1515, 1450, 1339, 1162, 820, 771, 735 cm^{-1}; ^{1}HNMR (200 MHz $CDCl_3$) δ 7.98 (s, 1H, C*H*=C), 7.75 (s, 1H, C*H*=N), 7.42-7.28 (m, 5H, Ar*H*), 5.30 (s, 2H, ArCH_2); CI-MS 203 (MH^+).

(2*S*)-2-(*N*-Benzyloxycarbonyl)amino-3-(5-benzyloxycarbonyl-1-imidazolyl) propionic Acid (11a) and (2*S*)-2-(*N*-Benzyloxycarbonyl)amino-3-(4-benzyloxycarbonyl-1-imidazolyl)propionic Acid (11b)

The procedure of Vederas and coworkers for the preparation of related compounds was followed.[67] To a solution of *N*-benzyloxycarbonyl-L-serine-β-lactone (0.288 g, 1.30 mmol) in 6.90 mL dry acetonitrile was added benzyl 4-imidazole carboxylate (**10**) (0.280 g, 1.38 mmol) in dry acetonitrile (7.40 mL). This was heated at 50 °C for 12 h and then the solvent was evaporated. Purification by reverse phase HPLC (C_{18} column) using 42% acetonitrile and 58% H_2O with 0.15% trifluoroacetic acid gave **11a** (0.071 g, 13%) and **11b** (0.053 g, 10%) in crystalline form.

For **11a**: mp 147 °C; IR (CH_2Cl_2 cast) 3300(m, br), 3035, 2960, 1727, 1671, 1525, 1455, 1410, 1335, 1194, 1139, 1062 cm^{-1}; ^1H NMR (300 MHz, d_6 acetone) δ 7.95 (s, 1H, C*H*=C), 7.78 (s, 1H, C*H* =N), 7.52-7.30 (m, 10H, Ar*H*), 5.35 (s, 2H, ArCH_2), 5.15- 5.05 (m, 1H, CH_2CH), 5.03 (s, 2H, ArCH_2), 4.88-4.78 (m, 1H, CH_2CH), 4.67-4.52 (q, 1H, C*H* CH_2), 4.49-4.0 (br, s, 1H, N*H*); MS (POS FAB, Cleland): 424 (MH^+).

For **11b**: mp 165-171 °C; IR (KBr) 3400 (m, br), 3040, 2980, 1730, 1660, 1595, 1528, 1458, 1410, 1340, 1200, 1142, 1068 cm^{-1}; ^1H NMR (400 MHz, d_6 acetone) δ 7.90 (s, 1H, C*H*=C), 7.73 (s, 1H, C*H* =N), 7.50-7.29 (m, 10H, Ar*H*), 5.27 (s, 2H, ArCH_2), 5.03 (s, 2H, ArCH_2), 4.68 (dd, 2H, *J* =8.0,16.0 Hz, CH_2 CH), 4.51 (q, 1H, *J* =8.0 Hz, C*H* NH), 4.37-3.45 (br, s, 1H, N*H*); MS (POS FAB, Cleland) 424 (MH^+) (423.418 calcd.for $C_{22}H_{21}N_3O_6$;); X-Ray crystallography confirmed the structure of **11b** (See the Appendix).

Methyl Chlorooximidoacetate (13)

The procedure of Kozikowski and Adamczyk for preparation of related compounds was adapted.[79] Glycine methyl ester hydrochloride (**12**) (0.500 g, 3.98 mmol) was dissolved in 2 mL of deionized H_2O and was treated with 0.333 mL of concentrated hydrochloric acid. The mixture was cooled to -5 °C, a solution of one equivalent of sodium nitrite (0.275

g, 3.98 mmol) in deionized H_2O (1 mL) was added dropwise, and the resulting solution was stirred for 10 min. A second equivalent of hydrochloric acid (0.333 mL) and a second equivalent of sodium nitrite (0.275 g, 3.98 mmol) were added slowly. The solution was stirred for 15 min and the reaction mixture was extracted with ethyl acetate (3 x 10 mL). The organic layer was dried over Na_2SO_4 and the solvent was removed *in vacuo* to give a yellow syrup. The crude syrup was crystallized from hexane to yield 0.060 g (21%) of **13** as white crystals: mp 65 °C; IR ($CHCl_3$ cast) 3500-3050, 1744, 1730, 1605 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 10.00–10.30 (br, s, 1H, NO*H*), 3.95 (s, 3H, C*H*$_3$O); CI-MS 137 (MH^+) ; Anal. Calcd. for $C_3H_4NO_3Cl$: C, 26.19; H, 2.93; N, 10.18; Cl, 25.77%. Found: C, 26.22; H, 3.09; N, 10.12; Cl, 24.86%.

Methyl 2-Amino-4-pentenoate Hydrochloride (19)

The procedure of Greenstein and Winitz for preparation of related compounds was adapted.[83] L-Allylglycine (**16**) (0.500 g, 4.34 mmol) was suspended in 15 mL of dry methanol and the solution was cooled to 0 °C. Dry hydrogen chloride gas was bubbled through the suspension. The amino acid dissolved rapidly. The hydrogen chloride treatment was continued until saturation of the solution (indicated by a 15% increase in the volume). The clear solution was concentrated *in vacuo* at 38 °C. To the resultant syrup was added 15 mL of dry methanol and this was subjected to a further hydrogen chloride saturation. The solvent was removed *in vacuo* to give a yellowish oil. This was dissolved in minimum volume of dry methanol and diethyl ether was added slowly to give a colourless oil. This was washed with diethyl ether and dried to yield 0.930 g (81%) of **19**: IR (CH_2Cl_2 cast) 3300-2700 (br), 2899, 1749 cm^{-1}; ^1H NMR (200 MHz, CD_3OD), δ 5.75-5.50 (m, 1H, C*H*CH_2), 5.20-5.05 (m, 2H, C*H*$_2$=CH), 4.01 (t, 1H, *J* = 4.0 Hz, C*H* NH_2), 3.65 (s, 3H, OC*H*$_3$), 2.60-2.38 (m, 2H, CHC*H*$_2$CH); CI-MS 130 (MH^+)

Methyl (2*S*)-2-(*N*-Benzyloxycarbonyl)amino-4-pentenoate (20)

The procedure of Nakajima and coworkers for preparation of related compounds was adapted.[84] To a stirred suspension of **19** (0.910 g, 5.61 mmol) in 15 mL $CHCl_3$ at -5 °C was added triethylamine (1.56 mL, 11.2 mmol) followed by slow addition of benzyl chloroformate (0.80 mL, 5.61 mmol) over 10 min. The solution was stirred for 3 h and then washed with 10% citric acid (15 mL) followed by 1M $NaHCO_3$ (15 mL) and deionized H_2O (15 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated *in vacuo* to give a crude syrup. Purification by silica gel flash chromatography (EtOAc/Hexane 25% / 75%) yielded 0.468 g (32%) of **20** as a colourless oil: IR ($CHCl_3$ cast) 3080-3000, 2980-2940, 1745, 1724, 1640, 1525 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.30-7.18 (m, 5H, Ar*H*), 5.71-5.49 (m, 2H, C*H*CH_2 & N*H*), 5.10-5.05 (m, 2H, CH_2=CH), 5.03 (s, 2H, ArCH_2), 4.39 (q, 1H, *J* =8.0 Hz, C*H*NH), 3.61 (s, 3H, OCH_3), 2.58-2.32 (m, 2H, CH_2 CH); CI-MS 264 (MH^+); Anal. Calcd. for $C_{14}H_{17}NO_4$: C, 63.86; H, 6.50; N, 5.32%. Found: C 63.30; H, 6.62; N, 5.08%.

Methyl (2*S*)-2-(*N*-Benzyloxycarbonyl)amino-3-(3-carbomethoxy-2-isoxazolin-5-yl) propionate (21a), Methyl (2*S*)-2-(*N*-Benzyloxycarbonyl)amino-3-(3-carbomethoxy-2-isoxazolin-5-yl)propionate (21b), Methyl (2*S*)-2-(*N*-Benzyloxycarbonyl)amino-3-(3-carbomethoxy-2-isoxazolin-4-yl)propionate (22a), and Methyl (2*S*)-2-(*N*-Benzyloxycarbonyl)amino-3-(3-carbomethoxy-2-isoxazolin-4-yl)propionate (22b)

The procedure of Kozikowski and Adamczyk for preparation of related compounds was adapted.[79] To a stirred solution of **13** (0.073 g, 0.532 mmol) in 2 mL of ether were added three equivalents of **20** (0.419 g, 1.59 mmol) in 3 mL of ether. To this stirred solution was added slowly over a period of 3 h, a solution of Na_2CO_3 (0.056 g, 0.532 mmol) in 2 mL of deionized H_2O, and vigorous stirring was continued for 12 h. The organic layer was separated, washed with deionized H_2O (10 mL), dried with Na_2SO_4, filtered and

concentrated *in vacuo* . The crude mixture was purified by silica gel flash chromatography (EtOAc 40% / hexane 60%) to give (as colorless oils) the separate epimers of the title compounds **21a** (0.079 g, 41%), **21b** (0.067 g, 35%), together with the minor regioisomers, the 4-substituted isoxazolines **22a** (3.82 mg, 2%) and **22b** (3.63 mg, 2%):

For **21a**: IR ($CHCl_3$ cast) 3040, 2960, 1745, 1722, 1590, 1525, 1440, 1350, 1210, 1128, 915 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.38-7.30 (m, 5H, Ar*H*), 5.70 (d, 1H, *J* =6.5 Hz, N*H*), 5.12 (s, 2H, ArCH_2), 5.0-4.85 (m, 1H, C*H*O), 4.55 (q, 1H, *J* =6.5 Hz, C*H*N), 3.85 (s, 3H, CH_3), 3.73 (s, 3H, CH_3), 3.28 (dd, 1H, *J* =10.0, 16.0 Hz, C*H*C=N), 2.89 (dd, 1H, *J* =8.0, 16.0 Hz, C*H* C=N), 2.18 (t, 2H, *J* =6.5 Hz, CH_2CHN); ^{13}C NMR (200 MHz, $CDCl_3$) δ 171.53 ($COOCH_3$), 160.65 ($COOCH_3$), 155.69 (*C*ONH), 151.02 (*C*=N), 135.89 (Quaternary aromatic *C*), 128.27-127.82 (Aromatic *C*H's), 80.01 (*C*ON), 66.82 (ArCH_2), 52.47 (CH_3), 51.05 (*C*HN), 38.66 (CH_2C=N), 37.17 (CH_2CHN); CI-MS 365 (MH^+); Anal. Calcd. for $C_{17}H_{20}N_2O_7$: C, 56.04; H, 5.53; N, 7.69%. Found: C, 55.30; H, 5.42; N, 7.38%.

For **21b**: IR (CH_2Cl_2 cast) 3360, 3040, 2960, 1740, 1723, 1589, 1525, 1439, 1344, 1258, 1218, 913 cm^{-1}; ^1H NMR (200MHz $CDCl_3$) δ 7.40-7.28 (m, 5H, Ar*H*), 5.62 (d, 1H, N*H*), 5.10 (s, 2H, ArCH_2), 4.95-4.78 (m, 1H, C*H*O), 4.58-4.44 (m, 1H, C*H*N), 3.85 (s, 3H, CH_3), 3.71 (s, 3H, CH_3), 3.28 (dd, 1H, *J* =10.0, 16.0 Hz, C*H*C=N), 2.84 (dd, 1H, *J* =8.0, 16.0 Hz, C*H*C=N), 2.28-2.12 (m, 1H, C*H*CHN), 2.08-1.89 (m, 1H, C*H*CHN); ^{13}C NMR (200 MHz, $CDCl_3$) δ 171.82 ($COOCH_3$), 160.80 ($COOCH_3$), 155.88 (*C*ONH), 151.26 (*C*=N), 135.98 (Quaternary aromatic *C*), 128.46-128.04 (Aromatic *C*H's), 80.70 (*C*ON), 67.07 (ArCH_2), 52.70 (CH_3), 51.66 (*C*HN), 38.95 (CH_2C=N), 37.53 (CH_2CHN); CI-MS 365 (MH^+); Anal. Calcd. for $C_{17}H_{20}N_2O_7$: C, 56.04; H, 5.53; N, 7.69%. Found: C, 55.69; H, 5.46; N, 7.55%.

For **22a**: IR ($CHCl_3$ cast) 3360, 3030, 2950, 1750, 1720, 1585, 1525, 1440, 1340, 1220, 940 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.40-7.32 (m, 5H, Ar*H*), 5.40 (d, 1H, *J* =8.0 Hz, N*H*), 5.10 (s, 2H, PhCH_2), 4.65-4.35 (m, 3H, CH_2ON & C*H*N), 3.86 (s, 3H, CH_3), 3.65 (s, 3H, CH_3), 3.67-3.48 (m, 1H, C*H*C=N), 2.27-2.11 (m, 1H, C*H*CHN), 2.09-1.90 (m, 1H, C*H*CHN); ^{13}C NMR (200 MHz $CDCl_3$) δ 128.60-128.16 (Aromatic *C's*), 76.74 (CH_2ON), 67.40 (ArCH_2), 52.82 (CH_3), 52.13 (*C*HN), 43.59 (*C*HC=N), 34.37 (CH_2CHN); CI-MS 365 (MH^+).

For **22b**: IR ($CHCl_3$ cast) 3360, 3035, 2960, 1740, 1720, 1585, 1520, 1440, 1340, 1220, 925 cm^{-1}; ^1H NMR (200 MHz, $CDCL_3$) δ 7.42-7.30 (m, 5H, Ar*H*), 5.43 (s, 2H, N*H*), 5.12 (s, 2H, ArCH_2), 4.60-4.31 (m, 3H, CH_2ON), 3.88 (s, 3H, CH_3), 3.78 (s, 3H, CH_3), 3.76-3.60 (m, 1H, C*H*C=N), 2.53-2.39 (m, 1H, C*H*CHN), 1.98-1.80 (m, 1H, C*H*CHN); ^{13}C NMR (200 MHz, $CDCL_3$) δ 128.60-128.20 (Aromatic *C's*), 76.74 (CH_2ON), 67.34 (ArCH_2), 52.78 (CH_3), 51.27 (*C*HN), 44.09 (*C*HC=N), 34.48 (CH_2CHN); CI-MS 365 (MH^+).

(2*S*)-2-(*N*-Benzyloxycarbonyl)amino-3-(3-carboxy-2-isoxazolin-5-yl) propionic Acid (23a)

The procedure of Vaughan and Spencer for the preparation of related compounds was adapted.[85] A mixture of compound **21a** (0.154 g, 0.424 mmol) and 10% sodium hydroxide (1.09 mL) was stirred at 20 °C for 3 h. Deionized H_2O (5 mL) was added and the mixture was stirred for 18 h. The mixture was washed with diethyl ether (10 mL). The aqueous layer was acidified with 10% hydrochloric acid and lyophilised to give a 0.214 g of crude mixture. Purification by reverse phase HPLC (aminopropyl column) gave 69 mg (48%) of **23a**: IR (KBr) 3418(s, br), 3040, 2940, 1716, 1701, 1628, 1527, 1410, 1341, 1262, 1057, 910 cm^{-1}; ^1H NMR (200 MHz, D_2O) δ 7.48-7.36 (m, 5H, Ar*H*), 5.15 (s, 2H, ArCH_2), 5.05-4.80 (m, 1H, C*H*O), 4.33 (t, 1H, *J* =6.0 Hz, C*H*N), 3.33 (dd, 1H, *J*

=10.0, 18.0 Hz, C*H*C=N), 2.97 (dd, 1H, *J* =8.0, 18.0 Hz, C*H*C=N), 2.18 (t, 2H, *J* =6.0 Hz, C*H*$_2$CHN); MS (POS FAB) 337 (MH^+), 359 (M^+Na), 381 $(M+2Na)^+$ (336.0957 calcd for $C_{15}H_{16}N_2O_7$).

(2*S*)-2-(*N*-Carbobenzyloxy)amino-3-(3-carboxy-2-isoxazolin-5-yl) propionic Acid (23b)

The procedure by Vaughan and Spencer for the preparation of related compounds was adapted.[85] A mixture of **21b** (0.174 g, 0.478 mmol) and 10% sodium hydroxide (1.22 mL) was stirred at 20 °C for 3 h. Deionized H_2O (5 mL)was added and the mixture was stirred for 18 h. The mixture was washed with diethyl ether (10 mL). The aqueous layer was then acidified with 10% hydrochloric acid and lyophilised to yield 0.344g crude mixture. Purification by reverse phase HPLC (aminopropyl column) gave 58 mg (36%) of isomer **23b**: IR (KBr) 3410(s, br), 3060, 2925, 1719, 1708, 1630, 1525, 1413, 1343, 1262, 1058, 922 cm^{-1}; ^1H NMR (200 MHz, D_2O) δ 7.32-7.22 (m, 5H, Ar*H*), 4.98 (s, 2H, ArC*H*$_2$), 4.78-4.60 (m, 1H, C*H*O), 4.17 (dd, 1H, *J* =4.0, 10.0 Hz, C*H*N), 3.15 (dd, 1H, *J* =10.0, 17.0 Hz, C*H*C=N), 2.73 (dd, 1H, *J* =8.0,17.0 Hz, C*H*C=N), 2.19-1.98 (m, 1H, C*H*CHN), 1.90-1.72 (m, 1H, C*H*CHN); MS (POS-FAB) 337 (MH^+), 359 $(M+Na)^+$, 381 $(M+2Na)^+$ (336.0957 calcd. for $C_{15}H_{16}N_2O_7$)

***o*-Nitrophenylsulfinyl Chloride (27)**

The procedure of Oae and Ikura for the preparation of related compounds was followed.[89] Bis(*o*-nitrophenyl)disulphide **(26)** (0.770 g, 2.49 mM) was mixed with two equivalent of glacial acetic acid (0.285 mL, 4.99 mM) in 5 mL of dry $CHCl_3$. The suspension was cooled to -5 °C and chlorine gas was added until no more chlorine was absorbed. The solvent was removed *in vacuo* to give a yellow solid. This was washed several times with $CHCl_3$ and the solvent was removed *in vacuo* to yield a yellow solid which was dried under vacuum to give 0.296 g (58%) of **27**: mp 52-56 °C; IR ($CHCl_3$ cast) 3100, 1590,

1563, 1545, 1491, 1447, 1323, 1301, 1106 cm^{-1}; ^1H NMR (200 MHz, $CHCl_3$) δ 8.40-8.30 (m, 1H, Ar*H*), 8.10–7.80 (m, 2H, Ar*H*), 7.50-7.45 (m, 1H, Ar*H*); CI-MS: 206 (MH^+)

3.3 Isolation of DAP epimerase

The procedure of Higgins *et. al.*[35] was modified. A frozen extract (80 g, previously sonicated) of DAP epimerase overproducing *Escherichia coli* LE134 was obtained as a gift from Dr. M. Pickard (Department of Microbiology, University of Alberta, Edmonton). The thawed extract was adjusted to 15% saturation with $(NH_4)_2SO_4$ and stirred. The cloudy solution so obtained was centrifuged at 12 000 x *g* for 1h to give a clear yellow supernatant and a brown pellet. The pellet was resuspended in a minimum amount of Buffer A (Buffer A consists of 20 mM potassium phosphate, 1mM EDTA and 1 mM dithiothreitol (DTT) pH 7.0) and dialysed three times against the same buffer. The ratio of the volume of resuspended pellet to the external buffer was 1:3. The supernatant was adjusted to 30% saturation with $(NH_4)_2SO_4$, which on centrifugation (12 000 x *g*, 1 h) gave a clear yellow supernatant and a small brown pellet. The pellet so obtained from 30% saturation was resuspended in a minimum amount of buffer A and dialysed against the same buffer (1:3 ratio by volume) while the supernatant was subjected to 45% saturation with $(NH_4)_2SO_4$. The pellet and the supernatant obtained from 45% saturation were treated in the same way as above. Assay of the various supernatants and the pellets, using the coupled enzyme assay, showed that most of the activity was in the 15% pellet and that the subsequent supernatants and pellets were devoid of activity by the time they were assayed.

The 15% pellet was a thick slurry which was diluted with 200 mL of buffer A and centrifuged at 17 500 x *g*. The clear supernatant obtained was assayed and substantial activity was observed in it. The pellet also showed some activity. The extraction of the

pellet was repeated using buffer A, until little activity was observed in the resulting supernatant. The combined supernatants showed a specific activity of 6.6 U/mg, where one unit of activity is defined as one µmol/min of NADPH formed under the condition of the coupled enzyme assay.

A 115 mL sample of the supernatant was loaded on a DE-52 cellulose column (5 cm x 18 cm) and the column was washed with 600 mL of buffer A followed by a gradient containing 0.0-0.25 M KCl in Buffer A. The fractions were assayed using the coupled enzyme assay, and those having similar specific activities were combined, dialysed against buffer A and concentrated by ultrafiltration to give two enzyme solution (25 mL each) with specific activities of 32 and 25 U/mg respectively.

The two solutions were combined and loaded on a Matrix™ Gel Green A affinity column (Amicon) (5 cm x 11 cm). The column was washed with 600 mL of buffer A and then with a gradient containing 0.0-1.6 M KCl in buffer A. The fractions having similar activities (assayed by coupled enzyme assay) were combined, dialysed against buffer A and concentrated by ultrafiltration to give two solutions (15 mL each) with specific activities of 79 U/mg and 32 U/mg.

3.4 Assays and inhibition studies

Coupled Enzyme Assay of DAP Epimerase

The method of Lam *et al* [46] was followed. The assays were performed at 25 °C in Buffer B (Buffer B consists of 0.1 M Tris-HCl, 1 mM EDTA and 1 mM dithiothreitol (DTT) at pH 7.8) using disposable cuvettes of a 1 cm light path. DAP epimerase activity was conveniently assayed by coupling the conversion of LL-DAP to *meso*-DAP with the $NADP^+$ -dependent oxidation of *meso*-DAP by *meso*-DAP dehydrogenase and then

following the increase in absorbance at 336-344 nm due to the formation of NADPH. The assay solution contained 0.3 mM $NADP^+$, 0.4 mM LL-DAP and 18 mU of DAP dehydrogenase in Buffer B. Aliquots of 5 μL of appropriately diluted fractions were added to 995 μL of the above assay solution to initiate the reaction. A unit of epimerase activity is defined as that which leads to the formation of 1 μmol/min NADPH under the conditions specified. The assay is linear over time for a change of up to 0.2 absorbance units under these conditions.

Assay of DAP Dehydrogenase

The method of Lam *et al* [46] was followed. The assays were performed at 25 °C in buffer B using disposable cuvettes of a 1 cm light path. The dehydrogenase activity was conveniently assayed by following the increase in absorbance at 336-344 nm with the formation of NADPH. The assay solution contained 0.3 mM $NADP^+$ and 0.33 mM *meso*-DAP in buffer B. A unit of activity is 1 μmol/ min of NADPH formed under conditions specified. The K_m for *meso*-DAP under these conditions is 0.11 mM.

Determination of Protein Content

The method of Bradford[94] was followed. Bio-Rad protein assay dye reagent and the standard assay conditions stated with this reagent were employed. Standard curves were prepared using bovine serum albumin diluted to the desired concentrations. Appropriately diluted enzyme solution (100 μL) was added to a test tube and 5 mL of (five fold diluted) Bio-Rad protein assay dye reagent was added to it. This mixture was vortexed and allowed to stand for 5 min. The absorbance was then measured at 594-596 nm against a blank solution which was prepared by the addition of 5 mL of (five fold diluted) dye reagent to 100 μL of deionized water.

Assay of DAP Analogues as Substrates of DAP Dehydrogenase

The assay was performed at 25 °C by replacing the *meso*-DAP in the standard assay solution by a 1.0 mM solution of the inhibitor and following any increase in absorbance at 336-344 nm. The assay solution contained 0.3 mM $NADP^+$ and 1.8 mU dehydrogenase in buffer B.

Assay of Substrate Analogues as Inhibitors of DAP Dehydrogenase

The one point control and inhibition study assays were performed at 25 °C in buffer B. The solution contained 0.3 mM $NADP^+$, 1.05 mM *meso*-DAP and 1.8 mU of dehydrogenase. For the inhibition studies a 1.0 mM solution of the inhibitor was added to the above assay solution.

Assay of Substrate Analogues as Inhibitors of DAP Epimerase Using the Coupled Enzyme Assay

The assay procedure described above was followed with the inclusion of substrate analogues (1.0 mM or 3.0 mM) in the assay solution.

Assay for Time Dependent Inhibition of DAP Epimerase with Substrate Analogues

Time dependent inhibition was determined by comparing the initial velocities of the control and inhibition curves obtained at different time intervals. The control curves were obtained by performing the assay at 25 °C in the same manner as described above, containing 0.3 mM $NADP^+$, 0.4 mM LL-DAP, 12.5 mU of epimerase and 1.8 mU of dehydrogenase. A sample containing the above was incubated and aliquots were drawn after 0, 20, 40 and 60 min time interval. The inhibition curves were also performed as above with the inclusion of 1.0 mM solution of the inhibitor.

Radioactive Assay of Substrate Analogues as Inhibitors of DAP Epimerase

A racemic mixture of tritiated DAP [G-^{3}H DAP] of specefic activity 1.09 mCi/mmol in H_2O (1 mL) (concentration 1 mCi/mL) was obtained from Amersham. To remove free tritiated water, an aliquot (100 μL) of the above solution was put in a vial and subjected to slow evaporation by a gentle stream of argon. Deionized H_2O (100 μL) was added to the resulting residue and evaporation was continued in the same manner. This process was repeated once more and the resulting residue was dissolved in deionized H_2O (1 mL). An aliquot (100 μL) was then taken and deionized H_2O (300 μL) was added to it to make a stock solution (0.017 μCi/μL). The radioactive assay procedure of Lam *et al.* [46] was adapted. The DAP epimerase activity was monitored by the epimerase-catalyzed release of ^{3}H to water from [G-^{3}H DAP] on incubation of the solution for 40 min at 25 °C. A run of total activity, blank, triplicates of control and triplicates of inhibitor at a given concentration were counted for the release of tritiated water. The assay mixture (100 μL) containing DAP epimerase (4.5 mU) in buffer B and diluted [G-^{3}H DAP] (0.5 μCi) was incubated for 40 min at 25 °C. The mixture was then quenched with 10% trichloroacetic acid, vortexed and applied to a column of ion exchange resin (0.5 ml, Bio-Rad AG50W-X8, H^+ form, 200-400 mesh). The eluant was collected, followed by washing of the column with deionized H_2O (3 x 500 μL) into a scintillation vial. Scintillation cocktail (10 mL) was then added to the above solution and this was counted in a scintillation counter for 15 min. Inhibitor runs were done by inclusion of inhibitor (0.5 mM) in the above reaction mixture.

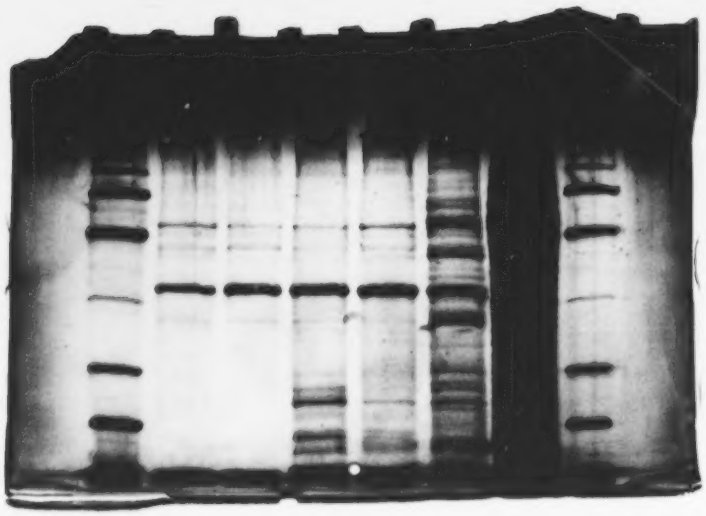


Figure 12: Electrophoresis gel of the purification steps of DAP epimerase.

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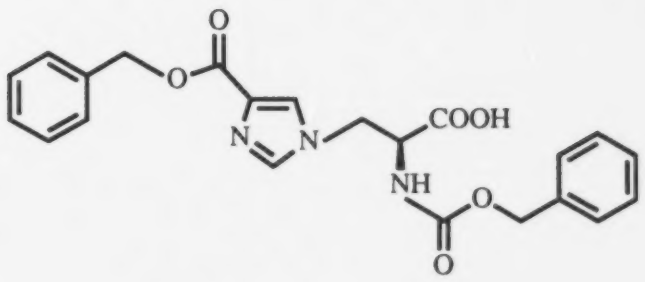
5. APPENDIX

5.1 Introduction

Title:

X-ray crystallography data of compound **11B**

Structure:



Crystallization Medium:

Acetone

Done By/At:

Dr. B.D.Santarsiero/ Molecular Structure Corporation, The Woodlands, Texas, USA

Experimental

DATA COLLECTION: All measurements were made on a Rigaku AFC5R diffractometer with graphite monochromated Cu K_α radiation anode generator.

Cell constants and an orientation matrix for data collection, obtained from a least-square refinement using the setting angles of 19 carefully centered reflections in the range $10.43 < 2\theta < 22.68$ ° corresponded to a monoclinic cell with dimensions:

a = 5.122 (3) A°

b = 9.093 (2) A° β = 94.17 (4) o

c = 23.276 (4) A°

v = 1081.3 (7) A°

STRUCTURE SOLUTION:..The structure was solved by direct methods.[1]

REFERENCE:

1. Structure solution methods:

MITHRIL

Gilmore, C.J.; *MITHRIL- an integrated direct methods computer program*; University of Glasgow, Scotland, *J. Appl. Cryst.* **1984**, *17*, 42-46.

DIRDIF

Beurskens, P.T.; DIRDIF: Direct Methods for Difference Structures - an automatic procedure for phase extension and refinement of difference structure factors. Technical Report 1984/1 Crystallography Laboratory, Toernooiveld, 6525 Ed Nijmegen, Netherlands.

Experimental Details

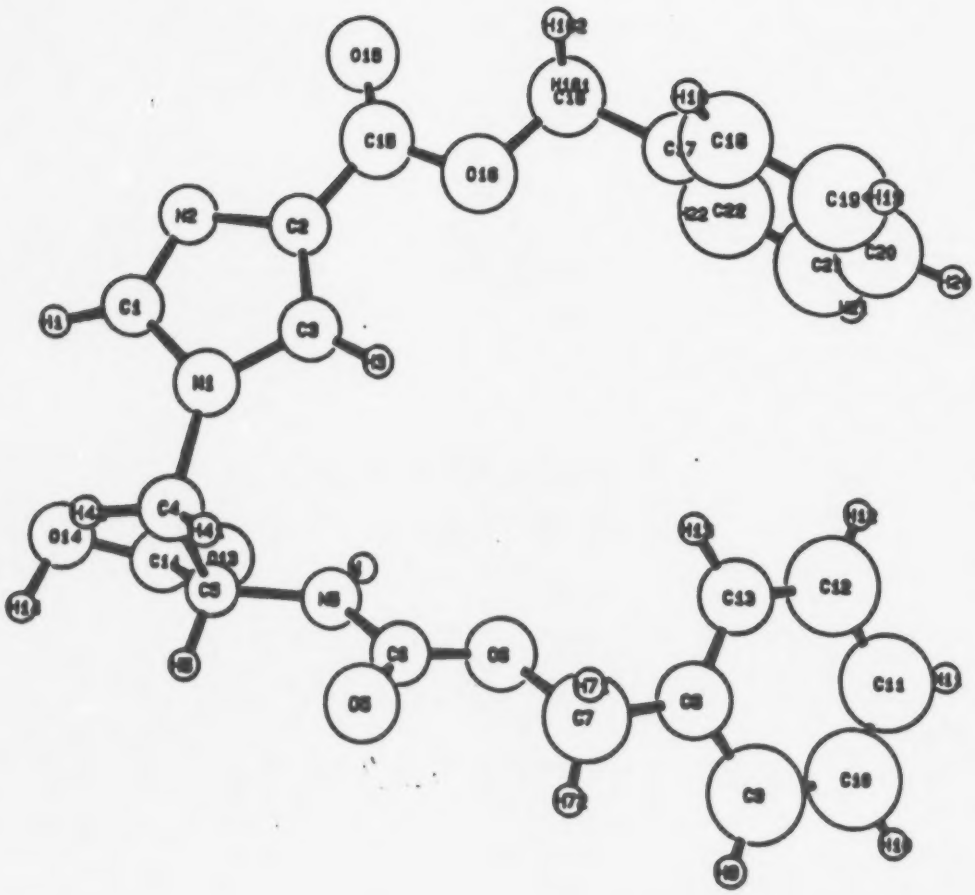
A. Crystal Data

Empirical Formula	$O_6N_3C_{22}H_{21}O_0$
Formula Weight	423.42
Crystal System	Monoclinic

B Intensity Measurements

Diffractometer	Rigaku AFC5R
Radiation	CuK_α (λ=1.54178)A^o

5.2. Data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C17
C16
C19
C22
C21
C20
N2
C2
C1
C3
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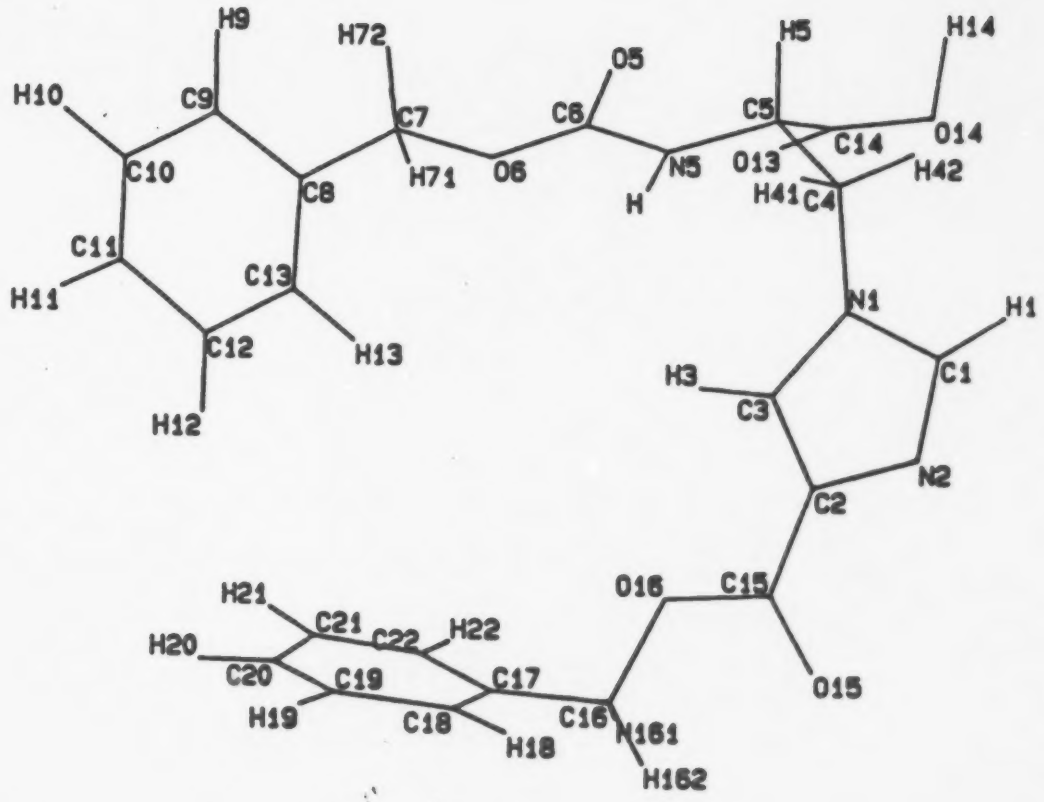
C7
C8
C13
C12
C11
C10
C9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O5
H5
H14
H10
C9
C7
C6
C5
O14
C14
C10
C8
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N5
O13
H42
H
H41
C4
C11
C13
N1
H1
H11
C12
H13
H3
C1
C3
H12
N2
C2
O16
C15
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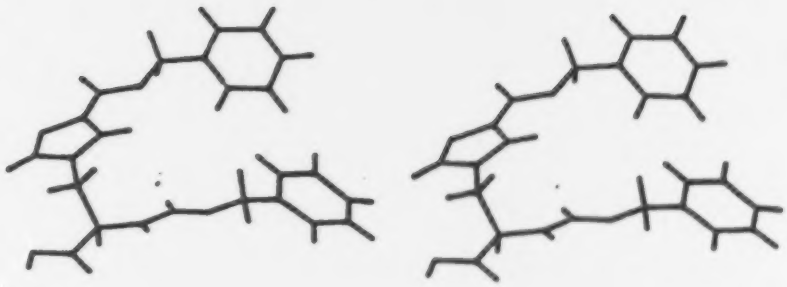
C17
O15
H19
C18
C16
H161
H18
H162



Atomic Coordinates

atom	x	y	z
O(5)	1.3709(24)	-0.0254	0.2150(05)
O(6)	1.0283(22)	-0.0281(18)	0.2719(05)
O(13)	0.5990(23)	0.1467(18)	0.1065(05)
O(14)	0.8284(20)	0.1059(18)	0.0305(05)
O(15)	0.2555(25)	-0.5566	0.0925(05)
O(16)	0.4827(22)	-0.5100(20)	0.1755(05)
N(1)	0.895(03)	-0.2163(20)	0.0843(06)
N(2)	0.5657(24)	-0.3335(20)	0.0399(05)
N(5)	0.9536(24)	0.0099(19)	0.1780(05)
C(1)	0.741(03)	-0.2352(24)	0.0324(07)
C(2)	0.598(04)	-0.3832(25)	0.0970(08)
C(3)	0.800(03)	-0.308(03)	0.1230(07)
C(4)	1.110(03)	-0.1021(24)	0.0922(07)
C(5)	1.019(03)	0.0322(22)	0.1202(07)
C(6)	1.137(03)	-0.0192(25)	0.2234(07)
C(7)	1.216(04)	-0.056(03)	0.3221(09)
C(8)	1.065(04)	-0.075(03)	0.3719(09)
C(9)	1.124(05)	0.009(03)	0.4188(13)
C(10)	0.985(06)	-0.010(04)	0.4678(11)
C(11)	0.800(05)	-0.102(04)	0.4703(12)
C(12)	0.744(06)	-0.196(04)	0.4236(12)
C(13)	0.882(04)	-0.178(03)	0.3750(08)
C(14)	0.794(04)	0.1048(25)	0.0834(08)
C(15)	0.431(04)	-0.491(03)	0.1180(09)
C(16)	0.329(04)	-0.619(03)	0.2036(09)
C(17)	0.408(04)	-0.622(03)	0.2666(08)
C(18)	0.607(05)	-0.699(03)	0.2867(11)
C(19)	0.680(05)	-0.695(04)	0.3486(13)
C(20)	0.542(05)	-0.615(03)	0.3800(10)
C(21)	0.347(06)	-0.525(04)	0.3609(12)
C(22)	0.276(05)	-0.533(04)	0.3025(12)
H	0.7745	0.0153	0.1859
H(1)	0.7631	-0.1839	-0.0025
H(3)	0.8638	-0.3185	0.1621
H(41)	1.2521	-0.1426	0.1154
H(42)	1.1665	-0.0768	0.0555
H(5)	1.1608	0.0996	0.1218
H(71)	1.3147	-0.1421	0.3159
H(72)	1.3318	0.0256	0.3279
H(9)	1.2590	0.0809	0.4185
H(10)	1.0322	0.0484	0.5008
H(11)	0.7017	-0.1066	0.5033
H(12)	0.6148	-0.2712	0.4251
H(13)	0.8434	-0.2402	0.3427
H(14)	0.9917	0.1711	0.0271
H(161)	0.1490	-0.5937	0.1981
H(162)	0.3575	-0.7125	0.1875
H(18)	0.7045	-0.7571	0.2618
H(19)	0.8252	-0.7493	0.3651
H(20)	0.5786	-0.6209	0.4205
H(21)	0.2640	-0.4598	0.3857
H(22)	0.1329	-0.4756	0.2869

Atomic Coordinates and B(eq)

atom	x	y	z	B(eq)
O(5)	1.3709(24)	-0.0254	0.2150(05)	5.9(3)
O(6)	1.0283(22)	-0.0281(18)	0.2719(05)	5.8(3)
O(13)	0.5990(23)	0.1467(18)	0.1065(05)	5.8(3)
O(14)	0.8284(20)	0.1059(18)	0.0305(05)	5.1(3)
O(15)	0.2555(25)	-0.5566	0.0925(05)	6.5(3)
O(16)	0.4827(22)	-0.5100(20)	0.1755(05)	6.7(3)
N(1)	0.895(03)	-0.2163(20)	0.0843(06)	4.5(4)
N(2)	0.5657(24)	-0.3335(20)	0.0399(05)	3.9(3)
N(5)	0.9536(24)	0.0099(19)	0.1780(05)	4.1(3)
C(1)	0.741(03)	-0.2352(24)	0.0324(07)	3.9(4)
C(2)	0.598(04)	-0.3832(25)	0.0970(08)	4.1(4)
C(3)	0.800(03)	-0.308(03)	0.1230(07)	4.2(4)
C(4)	1.110(03)	-0.1021(24)	0.0922(07)	4.4(4)
C(5)	1.019(03)	0.0322(22)	0.1202(07)	3.1(4)
C(6)	1.137(03)	-0.0192(25)	0.2234(07)	4.1(4)
C(7)	1.216(04)	-0.056(03)	0.3221(09)	8.4(6)
C(8)	1.065(04)	-0.075(03)	0.3719(09)	6.3(5)
C(9)	1.124(05)	0.009(03)	0.4188(13)	10.7(8)
C(10)	0.985(06)	-0.010(04)	0.4678(11)	11.5(8)
C(11)	0.800(05)	-0.102(04)	0.4703(12)	11.1(8)
C(12)	0.744(06)	-0.196(04)	0.4236(12)	11.3(9)
C(13)	0.882(04)	-0.178(03)	0.3750(08)	6.2(5)
C(14)	0.794(04)	0.1048(25)	0.0834(08)	4.9(5)
C(15)	0.431(04)	-0.491(03)	0.1180(09)	6.7(6)
C(16)	0.329(04)	-0.619(03)	0.2036(09)	7.1(6)
C(17)	0.408(04)	-0.622(03)	0.2666(08)	5.4(5)
C(18)	0.607(05)	-0.699(03)	0.2867(11)	9.0(7)
C(19)	0.680(05)	-0.695(04)	0.3486(13)	10.9(8)
C(20)	0.542(05)	-0.615(03)	0.3800(10)	8.9(7)
C(21)	0.347(06)	-0.525(04)	0.3609(12)	12.5(8)
C(22)	0.276(05)	-0.533(04)	0.3025(12)	10.8(7)
H	0.7745	0.0153	0.1859	5.0
H(1)	0.7631	-0.1839	-0.0025	4.7
H(3)	0.8638	-0.3185	0.1621	5.0
H(41)	1.2521	-0.1426	0.1154	5.2
H(42)	1.1665	-0.0768	0.0555	5.2
H(5)	1.1608	0.0996	0.1218	3.8
H(71)	1.3147	-0.1421	0.3159	10.0
H(72)	1.3318	0.0256	0.3279	10.0
H(9)	1.2590	0.0809	0.4185	12.9
H(10)	1.0322	0.0484	0.5008	13.9
H(11)	0.7017	-0.1066	0.5033	13.3
H(12)	0.6148	-0.2712	0.4251	13.7
H(13)	0.8434	-0.2402	0.3427	7.5
H(14)	0.9917	0.1711	0.0271	6.2
H(161)	0.1490	-0.5937	0.1981	8.5
H(162)	0.3575	-0.7125	0.1875	8.5
H(18)	0.7045	-0.7571	0.2618	10.8
H(19)	0.8252	-0.7493	0.3651	13.0
H(20)	0.5786	-0.6209	0.4205	10.6
H(21)	0.2640	-0.4598	0.3857	15.0
H(22)	0.1329	-0.4756	0.2869	13.0

Intramolecular Distances Involving the Nonhydrogen Atoms

atom	atom	distance
O(5)	C(6)	1.23(2)
O(6)	C(6)	1.29(2)
O(6)	C(7)	1.48(2)
O(13)	C(14)	1.23(2)
O(14)	C(14)	1.26(2)
O(15)	C(15)	1.20(2)
O(16)	C(15)	1.36(2)
O(16)	C(16)	1.45(2)
N(1)	C(1)	1.40(2)
N(1)	C(3)	1.35(2)
N(1)	C(4)	1.52(2)
N(2)	C(1)	1.29(2)
N(2)	C(2)	1.40(2)
N(5)	C(5)	1.43(2)
N(5)	C(6)	1.39(2)
C(2)	C(3)	1.34(2)
C(2)	C(15)	1.41(2)
C(4)	C(5)	1.48(2)
C(5)	C(14)	1.53(2)
C(7)	C(8)	1.45(2)
C(8)	C(9)	1.35(3)
C(8)	C(13)	1.33(2)
C(9)	C(10)	1.40(3)
C(10)	C(11)	1.27(4)
C(11)	C(12)	1.40(3)
C(12)	C(13)	1.39(3)
C(16)	C(17)	1.49(2)
C(17)	C(18)	1.30(3)
C(17)	C(22)	1.38(3)
C(18)	C(19)	1.46(3)
C(19)	C(20)	1.28(3)
C(20)	C(21)	1.34(3)
C(21)	C(22)	1.38(3)

Distances are in angstroms. Estimated standard deviations in the least significant figure are given in parentheses.

Intramolecular Distances Involving the Hydrogen Atoms

atom	atom	distance	atom	atom	distance
O(14)	H(14)	1.033	C(11)	H(11)	0.951
N(5)	H	0.950	C(12)	H(12)	0.950
C(1)	H(1)	0.951	C(13)	H(13)	0.950
C(3)	H(3)	0.950	C(16)	H(161)	0.950
C(4)	H(41)	0.950	C(16)	H(162)	0.949
C(4)	H(42)	0.950	C(18)	H(18)	0.950
C(5)	H(5)	0.950	C(19)	H(19)	0.950
C(7)	H(71)	0.950	C(20)	H(20)	0.949
C(7)	H(72)	0.950	C(21)	H(21)	0.952
C(9)	H(9)	0.950	C(22)	H(22)	0.949
C(10)	H(10)	0.949			

Distances are in angstroms. Estimated standard deviations in the least significant figure are given in parentheses.

Intramolecular Bond Angles Involving the Nonhydrogen Atoms

atom	atom	atom	angle	atom	atom	atom	angle
C(6)	O(6)	C(7)	114(1)	C(9)	C(8)	C(13)	118(2)
C(15)	O(16)	C(16)	117(2)	C(8)	C(9)	C(10)	120(3)
C(1)	N(1)	C(3)	107(2)	C(9)	C(10)	C(11)	123(3)
C(1)	N(1)	C(4)	123(1)	C(10)	C(11)	C(12)	119(3)
C(3)	N(1)	C(4)	130(1)	C(11)	C(12)	C(13)	118(3)
C(1)	N(2)	C(2)	108(1)	C(8)	C(13)	C(12)	122(2)
C(5)	N(5)	C(6)	124(1)	O(13)	C(14)	O(14)	127(2)
N(1)	C(1)	N(2)	109(1)	O(13)	C(14)	C(5)	119(2)
N(2)	C(2)	C(3)	107(2)	O(14)	C(14)	C(5)	113(2)
N(2)	C(2)	C(15)	121(2)	O(15)	C(15)	O(16)	121(2)
C(3)	C(2)	C(15)	131(2)	O(15)	C(15)	C(2)	129(2)
N(1)	C(3)	C(2)	108(2)	O(16)	C(15)	C(2)	110(2)
N(1)	C(4)	C(5)	112(1)	O(16)	C(16)	C(17)	110(2)
N(5)	C(5)	C(4)	114(1)	C(16)	C(17)	C(18)	121(2)
N(5)	C(5)	C(14)	112(1)	C(16)	C(17)	C(22)	118(2)
C(4)	C(5)	C(14)	111(1)	C(18)	C(17)	C(22)	121(2)
O(5)	C(6)	O(6)	128(2)	C(17)	C(18)	C(19)	119(2)
O(5)	C(6)	N(5)	120(2)	C(18)	C(19)	C(20)	118(3)
O(6)	C(6)	N(5)	112(1)	C(19)	C(20)	C(21)	126(3)
O(6)	C(7)	C(8)	107(2)	C(20)	C(21)	C(22)	116(3)
C(7)	C(8)	C(9)	119(2)	C(17)	C(22)	C(21)	121(3)
C(7)	C(8)	C(13)	123(2)				

Angles are in degrees. Estimated standard deviations in the least significant figure are given in parentheses.

Intramolecular Bond Angles Involving the Hydrogen Atoms

atom	atom	atom	angle	atom	atom	atom	angle
C(14)	O(14)	H(14)	104.56	C(11)	C(10)	H(10)	118.66
C(5)	N(5)	H	118.15	C(10)	C(11)	H(11)	120.40
C(6)	N(5)	H	118.13	C(12)	C(11)	H(11)	120.64
N(1)	C(1)	H(1)	125.61	C(11)	C(12)	H(12)	121.02
N(2)	C(1)	H(1)	125.65	C(13)	C(12)	H(12)	120.99
N(1)	C(3)	H(3)	125.77	C(8)	C(13)	H(13)	118.89
C(2)	C(3)	H(3)	125.74	C(12)	C(13)	H(13)	118.84
N(1)	C(4)	H(41)	108.88	O(16)	C(16)	H(161)	109.29
N(1)	C(4)	H(42)	108.91	O(16)	C(16)	H(162)	109.34
C(5)	C(4)	H(41)	108.90	C(17)	C(16)	H(161)	109.36
C(5)	C(4)	H(42)	108.93	C(17)	C(16)	H(162)	109.40
H(41)	C(4)	H(42)	109.46	H(161)	C(16)	H(162)	109.50
N(5)	C(5)	H(5)	106.64	C(17)	C(18)	H(18)	120.81
C(4)	C(5)	H(5)	106.61	C(19)	C(18)	H(18)	120.58
C(14)	C(5)	H(5)	106.61	C(18)	C(19)	H(19)	121.28
O(6)	C(7)	H(71)	110.04	C(20)	C(19)	H(19)	120.94
O(6)	C(7)	H(72)	110.06	C(19)	C(20)	H(20)	117.25
C(8)	C(7)	H(71)	109.96	C(21)	C(20)	H(20)	116.92
C(8)	C(7)	H(72)	109.98	C(20)	C(21)	H(21)	122.32
H(71)	C(7)	H(72)	109.48	C(22)	C(21)	H(21)	122.00
C(8)	C(9)	H(9)	120.08	C(17)	C(22)	H(22)	119.29
C(10)	C(9)	H(9)	120.30	C(21)	C(22)	H(22)	119.46
C(9)	C(10)	H(10)	118.42				

Angles are in degrees. Estimated standard deviations in the least significant figure are given in parentheses.

Table of Least-Squares Planes

---------- Plane number 1 ----------

Atoms Defining Plane	Distance	esd
N(1)	0.0019	0.0156
C(1)	0.0001	0.0182
N(2)	-0.0018	0.0150
C(2)	0.0048	0.0196
C(3)	-0.0049	0.0188

Additional Atoms	Distance
C(4)	-0.0821
C(15)	-0.0060

Mean deviation from plane is 0.0027 angstroms
Chi-squared: 0.2

---------- Plane number 2 ----------

Atoms Defining Plane	Distance	esd
C(8)	-0.0169	0.0209
C(9)	0.0128	0.0269
C(10)	0.0167	0.0308
C(11)	-0.0301	0.0305
C(12)	0.0113	0.0302
C(13)	0.0096	0.0212

Additional Atoms	Distance
C(7)	0.0154

Mean deviation from plane is 0.0162 angstroms
Chi-squared: 2.5

Dihedral angles between least-squares planes

plane 2	plane 1	angle
		36.90

Table of Least-Squares Planes (continued)

---------- Plane number 3 ----------

Atoms Defining Plane	Distance	esd
C(17)	-0.0155	0.0221
C(18)	0.0165	0.0262
C(19)	0.0115	0.0286
C(20)	-0.0314	0.0273
C(21)	0.0329	0.0310
C(22)	0.0055	0.0285

Additional Atoms	Distance
C(16)	0.0253

Mean deviation from plane is 0.0189 angstroms
Chi-squared: 3.6

Dihedral angles between least-squares planes

plane	plane	angle
3	1	94.87
3	2	97.71